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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{6} :
A61K 49/00, 41/00

A1

(11) International Publication Number: **WO 98/48845**

(43) International Publication Date: 5 November 1998 (05.11.98)

(21) International Application Number: PCT/GB98/01245

(22) International Filing Date: 28 April 1998 (28.04.98)

(30) Priority Data:

08/848,586	29 April 1997 (29.04.97)	US
9727124.1	22 December 1997 (22.12.97)	GB
09/035,285	5 March 1998 (05.03.98)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications
US 08/848,586 (CIP)
Filed on 29 April 1997 (29.04.97)
US 09/035,285 (CIP)
Filed on 5 March 1998 (05.03.98)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD OF DEMARCATING TISSUE

(57) Abstract

This invention provides a method of treatment of the human or animal body to remove tumorous tissue therefrom, wherein an effective amount of a dye compound is administered to said body and allowed to accumulate at tumorous tissues or cells therein and wherein tumorous tissue or cells at which said dye compound has accumulated is removed or destroyed in situ, the improvement comprising using as said dye compounds a physiologically tolerable dye compound being a polysulphonic acid the molecular structure whereof comprises at least one chromophore and at least 5 sulphonic acid groups, or a derivative thereof.

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Method of Demarcating Tissue

Field of the Invention

This invention relates to the use of dye compounds in surgical procedures, and in particular to the use of dye compounds to delineate tumor margins in surgery to remove tumors and in photodynamic therapeutic techniques.

Background to the Invention

It is often found that tumors are similar in colour and in texture to the healthy tissue in which they are embedded. Additionally, tumors often have irregular borders. However, since tumors are able to metastasize, the location, identification and subsequent removal of associated tumors and diseased lymph nodes is as important as the removal of the primary tumor. Therefore, the ability to be able to clearly differentiate tumorous tissue from the surrounding benign tissue is important in that it will greatly facilitate the tumor's complete surgical excision, whilst at the same time minimising the amount of benign tissue removed. This latter factor is frequently an important factor in producing a successful surgical outcome and thus increasing the patient's quality of life and/or chances of long-term survival.

It has been found that a number of techniques including magnetic resonance imaging (MRI), ultrasound imaging and X-ray (e.g., CT) imaging can define both the location and approximate size of a tumor. However, all of these techniques are either not useful or unduly awkward for real-time guidance during actual surgery, and additionally are not as precise as direct visualisation.

The use of dyes in medicine has a long history. Their first uses were in attempts to kill microorganisms in water, staining tissues in the field of histology and later as diagnostic aids.

The importance of fluorescence has always been recognised, both for its enhanced visualisation properties and also for its association with disinfection. It was first reported by Policard et al. in Compt. Rend. Soc. Biol. 91 (1924) 1423 that both animal and human tumors could exhibit a red fluorescence, this being attributed to the accumulation of endogenous porphyrins. More recently Figge et al. in Proc. Soc. Exp. Biol. Med. 68 (1948) 600, observed that certain porphyrin derivatives preferentially localized in tumors and thus considerable efforts have subsequently been directed towards the use of dyes, especially hematoporphyrin derivatives, as the agents of photodynamic therapy (PDT) for the treatment of tumors (see Kessel [Ed.], in Photodynamic Therapy of Neoplastic Disease (2 volumes) CRC Press (1990)). In this technique the aim is to selectively stain tumors with dyes and subsequently expose them to light. This results, with varying degrees of success, in the destruction of the tumor.

An alternative approach to tumor therapy is provided by recognising that the ability to selectively stain tumors with a dye would be a valuable aid in their subsequent surgical removal (see Moore in Science 106 (1947) 130 where fluorescein was used to aid in the location of gastric carcinomas). In addition to fluorescein, methylene blue was frequently used, often to stain the sometimes difficult to locate parathyroid glands (see Wheeler et al., in Amer. J. Surg 143 (1982) 713). Fluorescein continues to find use, for example see Googe et al. in Ophthalmology 100 (1993) 1167 wherein its use in angiography is described. On the other hand, hematoporphyrin derivative, a first generation PDT agent obtained from a chemical treatment of hematoporphyrin, has also been proposed for the diagnosis of cancer when used with infrared fluorescence detection (see Profio in IEEE J. Quant. Elect. OE-20 (1984) 1502).

Other classes of dye used include porphyrin derivatives, for example see Takemura et al. in Photochem. Photobiol. 59 (1994) 366, and indocyanine green (ICG), a cyanine dye, that has been used for human gliomas, for example see Haugland et al. in Neurosurgery 38 (1996) 308.

The following listed patents describe dyes previously disclosed in this field and are herein incorporated by reference: WO-A-96/17628, US-A-5672332, WO-A-97/18841, WO-A-96/23525, WO-A-95/20981 and WO-A-95/26754.

It has recently been found that the use of dyes both absorbing and fluorescing at wavelengths between 300 and 1300 nm, is particularly advantageous, tissue penetration being greater at these wavelengths than with shorter or longer wavelengths. For example Poon et al. in J. Neurosurg. 76 (1992) 679 details an intraoperative investigation using chloroaluminium phthalocyanine tetrasulfonate ($ClAlPcS_4$) which fluoresces at 680 nm, for the delineation of rat gliomas. This compound, and other phthalocyanines, have been frequently investigated and shown to possess useful localising properties for PDT. However, their use has not yet achieved wide acceptance in practice.

In addition, it is possible to make use of modern inexpensive semiconductor diode laser dyes that emit in this wavelength region. Most importantly, these wavelengths are harmless to the human body. Dyes which fall into this category often have high extinction coefficients ($\epsilon \geq 10^5 M^{-1} cm^{-1}$), thus rendering tissues stained by them readily visible for traditional or laser surgery, consequently facilitating removal of the bulk of the tumor. The fluorescence of the dye can then be used to visualise any residual small tumor fragments which can then also be removed due to fluorescence being readily detected by the human eye.

It may also be desirable to destroy residual stained tumor fragments by exposing the surgical site after removal of the tumor with an appropriate dose of light radiation at or near the absorption maximum of the dye. This process is known as post-surgical PDT. A number of recent studies have described the intraoperative use of PDT for cleaning up a tumor removal site after surgery, for example van Hillegersberg et al. in Drugs 48 (1994) 510.

To improve detection of the tumors, it is desirable for the dyes to localise at the tumor site in an amount adequate for ready visualisation and that they are retained in adjoining healthy tissues to a significantly lower extent so that the two tissue types can readily be distinguished. Furthermore, the dyes should preferably possess high quantum yields of fluorescence in the presence of bodily fluids, thus enabling even small amounts of absorbed dyes to generate a readily visible fluorescence signal.

Laser surgery is particularly advantageous when used in conjunction with tumor localizing dyes, since the laser wavelength can be chosen to be close to the absorption wavelength of the dye, enabling stained tumorous tissues to be vaporized without significant damage to adjacent unstained normal tissues.

Dyes may be actively targeted to a tumor. Active targetting is defined as a modification of biodistribution using chemical groups that will associate with species present in the tumor tissue to effectively decrease the rate of loss of dye from the tumor tissue. In such cases the dye is synthetically modified in order to facilitate its location at a specific tumor site. Alternatively, dyes may passively distribute (i.e. passively target) into tumors which means that, even without synthetic manipulation, they tend to accumulate in tumorous tissues.

To date, no dye has been shown to sufficiently combine the various properties such as adequate localization on a convenient timescale, necessary for wide acceptance for intraoperative use.

Summary of the Invention

It has now been found that dye compounds can be used effectively to delineate tumor margins or to pinpoint tumorous tissue or cells or diseased lymph nodes where the dye compound (i) is presented in particulate form, (ii) is a polychromophore containing at least one sulphonic acid group, (iii) is a cofacial polychromophore, preferably containing at least one sulphonic acid group, (iv) is a vector-attached chromophore or polychromophore, (v) is a polysulphonic acid compound containing at least 5 sulphonic acid groups or derivatives thereof, (vi) is a polychromophore, the chromophoric groups of which are linked together by a polymer, preferably by polyalkyleneoxy groups, preferably a sulphonic acid group containing polychromophore, (vii) is a polymer or surfactant molecule to which chromophoric groups have been attached, or (viii) meets two or more of the foregoing criteria.

Thus viewed from one aspect the invention provides a method of treatment of the human or animal (e.g., mammalian, avian or reptilian) body to remove tumorous tissue therefrom, wherein an effective amount of a dye compound is administered to said body and allowed to accumulate at tumorous tissues or cells therein and wherein tumorous tissue or cells at which said dye compound has accumulated is removed or destroyed in situ, the improvement comprising using as said dye compound a compound or compound mixture which (i) is administered in particulate form, (ii) is a polychromophore containing at least one sulphonic acid group, (iii) is a cofacial polychromophore, preferably containing at least one sulphonic acid group, (iv) is a vector-attached chromophore or polychromophore, (v) is a polysulphonic acid compound containing at least 5 sulphonic acid groups or derivatives thereof, (vi) is a polychromophore, the chromophoric groups of which are linked together by a polymer, preferably by polyalkyleneoxy groups, preferably a sulphonic acid group containing polychromophore, (vii) is a polymer or surfactant molecule to which chromophoric groups have been attached, or (viii) meets two or more of the foregoing criteria.

It should be appreciated that the method of treatment described above may be such that the distance to the surface of said body where the dye compound accumulates is such that said dye compound may be visible at the surface of said body. In this manner the dye compound may be external to said body or, alternatively internal to said body but exposed by surgery etc.

Viewed from a further aspect the invention provides the use of a physiologically tolerable dye compound for the manufacture of a medicament in which said dye compound (i) is present in particulate form, (ii) is a polychromophore containing at least one sulphonic acid group, (iii) is a cofacial polychromophore, preferably containing at least one sulphonic acid group, (iv) is a vector-attached chromophore or polychromophore, (v) is a polysulphonic acid compound containing at least 5 sulphonic acid groups or derivatives thereof, (vi) is a polychromophore, the chromophoric groups of which are linked together by a polymer, preferably by polyalkyleneoxy groups, preferably a sulphonic acid group containing polychromophore, (vii) is a polymer or surfactant molecule to which chromophoric groups have been attached, or (viii) meets two or more of the foregoing criteria, for use in a method of treatment of the human or animal body to remove tumorous tissue or cells therefrom or to destroy tumorous tissue or cells therein.

The polysulphonate and sulphonated polychromophore dye compounds, the vector-attached chromophore (and polychromophore) compounds and the polyalkylenoxy-linked polychromophores are themselves novel and thus viewed from a further aspect the invention provides a composition of matter comprising a physiologically tolerable dye which (i) is a polychromophore compound containing at least one sulphonic acid group, (ii) is a polysulphonic acid compound containing at least 5 sulphonic acid groups or derivatives thereof, (iii) is a chromophore (or polychromophore) coupled to a vector with affinity for tumor tissue, tumor cells or diseased lymph nodes, (iv) is a polychromophore, the chromophoric groups of which are linked together by a polymer, preferably by polyalkyleneoxy groups, preferably a sulphonic acid group containing polychromophore, (v) is a polymer or surfactant molecule to which chromophoric groups have been attached, or (vi) meets two or more of the foregoing criteria, e.g., for use in surgery.

Viewed from a yet further aspect the invention provides a pharmaceutical composition comprising a physiologically tolerable dye which (i) is a polychromophore compound containing at least 5 sulphonic acid groups or derivatives thereof, (ii) is a polysulphonic acid compound containing at least 5 sulphonic acid groups or derivatives thereof, (iii) is a chromophore (or polychromophore) coupled to a vector with affinity for tumor tissue, tumor cells or diseased lymph nodes, (iv) is a polychromophore, the chromophoric groups of which are linked together by a polymer, preferably by polyalkyleneoxy groups, preferably a sulphonic acid group containing polychromophore, (v) is a polymer or surfactant molecule to which chromophoric groups have been attached, or (vi) meets two or more of the foregoing criteria, together with at least one physiologically acceptable carrier or excipient, e.g., in a sterile, pyrogen free aqueous carrier medium.

Thus viewed from a further aspect the invention provides a method of treatment of the human or animal (e.g., mammalian, avian or reptilian) body to remove tumorous tissue therefrom, wherein an effective amount of a dye compound is administered to said body and allowed to accumulate at tumorous tissues or cells therein and wherein tumorous tissue on cells at which said dye compound has accumulated is removed or destroyed in situ, the improvement comprising using as said dye compound a physiologically tolerable dye compound the molecular structure whereof comprises at least one chromophore and at least 5 sulphonic acid groups or a derivative (e.g., a salt) thereof.

Viewed from a further aspect the invention provides the use of a physiologically tolerable dye compound the molecular structure whereof comprises at least one chromophore and at least 5 sulphonic acid groups or a derivative (e.g., a salt) thereof for the manufacture of a medicament for use in a method of treatment of the human or animal body to remove tumorous tissue or cells therefrom or to destroy tumorous tissue or cells therein.

Thus viewed from a further aspect the invention provides a method of imaging of the human or non-human animal body, which comprises administering to said body a light imaging contrast agent and generating by a light imaging modality an image of at least a part of said body to which said agent distributes, the improvement comprising administering as said agent a dye compound or compound mixture which (i) is administered in particulate form, (ii) is a polychromophore containing at least one sulphonic acid group, (iii) is a cofacial polychromophore, preferably containing at least one sulphonic acid group, (iv) is a vector-attached chromophore or polychromophore, (v) is a polysulphonic acid compound containing at least 5 sulphonic acid groups or derivatives thereof, (vi) is a polymer or surfactant molecule to which chromophoric groups have been attached, or (vii) meets two or more of the foregoing criteria, said agent being a material which accumulates at a tumorous lesion, tumor cells or diseased lymph nodes and said part of said body being a part containing or suspected to contain or at risk of containing a tumorous lesion, tumor cells or a diseased lymph node.

As used in this application, "light imaging" refers to diagnostic procedures that result in an image of the interior of the body or of the surface of the body by a process involving propagation through or reflection from the body of electromagnetic radiation with a wavelength in the range 300 to 1300 nm. The propagating electromagnetic radiation can result either from irradiation of the body or a portion of the body with light in the specified wavelength range or by generation of light within the wavelength range in the body or on the surface of the body by processes such as bioluminescence, sonoluminescence, or fluorescence.

The propagating light can be detected directly with sensors at the surface or within the body or indirectly after the generation from the light of another form of energy such as sound, ultrasound, heat, or electromagnetic radiation outside of the wavelength range 300 to 1300 nm. A sensor is a device that converts the detected light, sound, ultrasound, heat or other form of signal into a recordable form such as electrical current. An example of a light sensor is a photomultiplier tube. A sensor for light may also consist of a fiber-optic cable leading from the point of detection to a photomultiplier tube. In a simple embodiment of light imaging the sensor consists of the human eye.

Viewed from a yet further aspect the invention provides the use of a physiologically tolerable dye compound or compound mixture which (i) is administered in particulate form, (ii) is a polychromophore containing at least one sulphonic acid group, (iii) is a cofacial polychromophore, preferably containing at least one sulphonic acid group, (iv) is a vector-attached chromophore or polychromophore, (v) is a polysulphonic acid compound containing at least 5 sulphonic acid groups or derivatives thereof, (vi) is a polymer or surfactant molecule to which chromophoric groups have been attached, or (vii) meets two or more of the foregoing criteria, for the manufacture of a contrast medium for use in a method of treatment or diagnosis which involves administering said contrast medium to a subject an generating by a light imaging modality an image of a part of said subject containing or suspected to contain or at risk of containing a tumorous lesion, tumor cells or a diseased lymph node.

Viewed from a yet still further aspect the invention provides a method of imaging of the human or non-human animal body, preferably an animate body, which comprises administering to said body a light imaging contrast agent and generating by a light imaging modality an image of at least a part of said body to which said agent distributes, the improvement comprising using as said modality a confocal scanning laser microscopy (CSLM) or optical coherence tomography (OCT) technique.

Viewed from a yet further aspect the invention also provides the use of a physiologically tolerable particulate or chromophore containing material, e.g., a chromophoric material as described herein, for the manufacture of a contrast medium for use in a method of treatment or diagnosis of a, preferably animate, human or non-human animal body which involves generation by a CSLM or OCT technique an image of a part of said body to which said material distributes.

Where the dye compound used according to the invention is a polychromophore or is used in particulate form, it preferably carries a plurality, i.e., at least 2, more preferably at least 4 and even more preferably at least 5, sulphonic acid groups, for example bound to the chromophores or to non-chromophoric linking groups or exposed on the surface of the particles.

The polysulphonate dye compounds used according to the invention are themselves novel and thus viewed from a further aspect the invention provides a physiologically tolerable dye compound being a polysulphonic acid the molecular structure whereof comprises at least one chromophore and preferably at least 5 sulphonic acid groups or a derivative (e.g., a salt) thereof.

Viewed from a still further aspect, the invention provides a pharmaceutical composition comprising a physiologically tolerable dye compound being a polysulphonic acid the molecular structure whereof comprises at least one chromophore and preferably at least 5 sulphonic acid groups or a derivative (e.g., a salt) thereof, together with at least one physiologically tolerable carrier or excipient, e.g., a composition comprising such a dye compound in a sterile, pyrogen free aqueous carrier medium.

Where the dye compound used according to the invention is administered in particulate form, this is preferably: (i) a nanoparticulate form having a mean particle size of up to 100 nm, e.g., 2 to 90 nm, preferably 8 to 80 nm; (ii) a liposomal form with the dye compound within the liposome core or associated with the liposomal membrane; (iii) a particulate form having surface sulphonic acid groups; (iv) a droplet form (i.e., an oil-in-water emulsion); or (v) in a form satisfying two or more of the foregoing criteria.

Nanoparticles, e.g., of a water-insoluble dye compound or containing or coated with a dye are a preferred format due to their ability to passively target tumor tissue and lymph nodes.

Combinations of dye can be used in the methods of the present invention, wherein the dyes used can be the same or different chemical structures.

Detailed Description of the Invention

As mentioned above, one class of dye compounds used according to the invention contain at least one chromophore and preferably at least five sulphonic acid groups (or derivatives thereof, e.g., salts).

Where the compounds contain more than one chromophore, these may be the same or different. Generally however it will be preferred that the chromophores are the same.

The dye compounds used according to the invention can either be used alone or in combination with other dyes. In addition, it is possible to use combinations of different dye compounds according to the invention. Suitable dye compounds for use in this invention are disclosed in the copending PCT application of Nycomed Imaging AS, filed 29 April 1998.

The chromophores will preferably be fluorophores, i.e., the compounds of the invention are preferably fluorescent. In this way they are easier to detect by eye or using appropriate equipment during surgery.

Particularly preferably, the chromophores will have absorption maxima in the region of 300-1300 nm, especially 650 to 1200 nm, and more especially 650 to 900 nm. Appropriate chromophores are well known and can readily be modified to carry sulphonic acid groups, these groups preferably being disposed about the chromophore rather than all being attached in the same general area of the molecule. Preferably, sulphonic acid groups will be attached to aryl groups in the chromophores either directly or via C_{1-10} linkers such as alkylene chains.

Preferred chromophores include compounds having an extensive delocalized electron system, e.g., cyanines, merocyanines, phthalocyanines, naphthalocyanines, triphenylmethanes, porphyrins, pyrylium dyes, thiapyrylium dyes, squarylium dyes, croconium dyes, azulenium dyes, indoanilines, benzophenoxazinium dyes, benzothiaphenothiazinium dyes, anthraquinones, naphthoquinones, indathrenes, phthaloylacridones, trisphenoquinones, azo dyes, intramolecular and intermolecular charge-transfer dyes and dye complexes, tropones, tetrazines, bis(dithiolene) complexes, bis(benzene-dithiolate) complexes, iodoaniline dyes, bis(S,O-dithiolene) complexes, etc. Examples of suitable organic or metallated organic chromophores may be found in "Topics in Applied Chemistry: Infrared absorbing dyes" Ed. M. Matsuoka, Plenum, NY 1990, "Topics in Applied Chemistry: The Chemistry and Application of Dyes", Waring et al., Plenum, NY, 1990, "Handbook of Fluorescent Probes and Research Chemicals" Haugland, Molecular Probes Inc, 1996, DE-A-4445065, DE-A-4326466, JP-A-3/228046, Narayanan et al. J. Org. Chem. 60: 2391-2395 (1995), Lipowska et al. Heterocyclic Comm. 1: 427-430 (1995), Fabian et al. Chem. Rev. 92: 1197 (1992), WO96/23525, Strekowska et al. J. Org. Chem. 57: 4578-4580 (1992), WO (Axis) and WO96/17628. Particular examples of visible-region chromophores which may be used include xylene cyanole, fluorescein, dansyl, NBD, Pc, indocyanine green, DODCI, DTDCI, DOTCI, DTTCI and DDTCI.

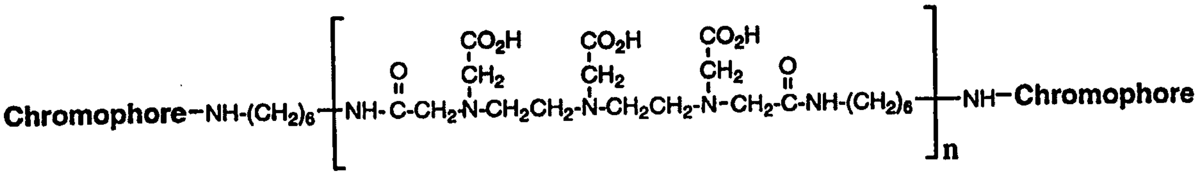
Particularly preferred are compounds which have absorption maxima between 600 and 1000 nm to avoid interference with haemoglobin absorption (e.g., xylene cyanole).

Another class of contrast agents that falls within the scope of this invention includes chromophore-bearing polymeric ligands for metal ions. One useful subset of this class is comprised of polymeric ligands which are particularly effective for complexing lanthanides and other metals used in magnetic resonance imaging. Polymeric ligands and their metal complexes, without chromophores attached, which are particularly useful in this application are described in U.S. Patent Application 08/478,803, U.S. Patent 5,583,206, and World Patent Application 96/40274. These and analogous polymers can be structurally modified to incorporate chromophore moieties, either subsequent to their synthesis or in a modification of the synthetic process used to prepare them. Included in those patent references are ranges of ligands and metals which are also preferred in the present invention. In addition, the metal ions of the current invention can include radionuclides. Also note that in the current invention, the degree of metal chelation can range from zero percent to one hundred percent of the theoretical maximum amount of metal ion which can be chelated by the chelator.

While chromophores can be attached directly to such polymeric ligands and complexes, modification of the polymer synthesis by increasing the proportion of the bis-amine monomer in the polymerization step can be done to provide closely related polymers which are primarily terminated by amines. These intermediates can then be reacted with chromophore molecules bearing amine-reactive attachment sites to synthesize polymeric ligands or complexes bearing chromophores.

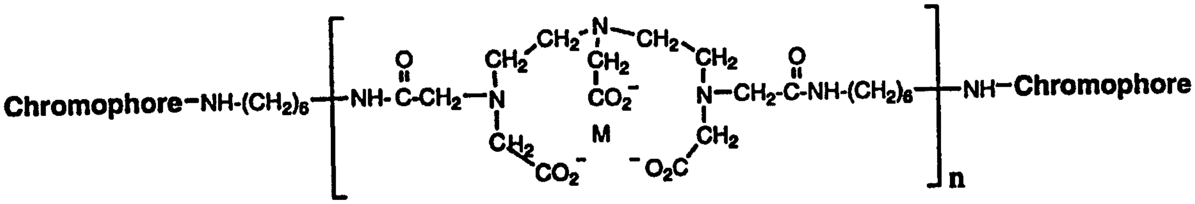
In polymeric MRI chelatore and complexes which also bear chromophores, the chromophores can be attached to termini of the polymer backbone or its branches. The attachment can also be at sites within the polymer backbone or of its branches. Two or more chromophores are attached to each polymer molecule. The ligand can be uncomplexed or complexed with a metal cation. Depending on the charge of the metal ion and the nature of the ligand there may also be counterions associated with the complex to provide charge neutrality. Counterions may also be associated with the carboxylic acids of the uncomplexed or partially complexed ligand polymer. Meglumine is particularly desirable for incorporation as a counterion, although any of the counterions described in the above patents could also be employed.

An example of such a chromophore bearing ligand is illustrated below, wherein n is defined as an integer whose value causes the molecular weight of the polymer to fall within the molecular weight range defined in the present invention:

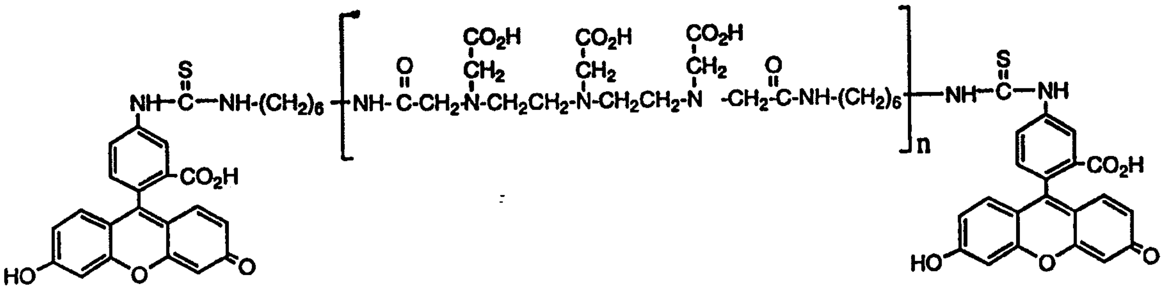


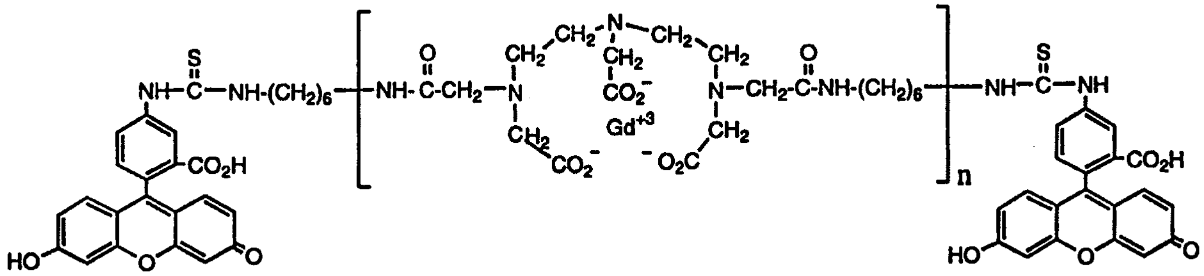
$$\text{Chromophore-NH-}(CH_2)_6\left[\text{NH-}\overset{O}{\overset{\|}{C}}\text{-}CH_2\text{-}\overset{\overset{CO_2H}{|}}{\overset{CH_2}{\overset{|}{N}}}\text{-}CH_2CH_2\text{-}\overset{\overset{CO_2H}{|}}{\overset{CH_2}{\overset{|}{N}}}\text{-}CH_2CH_2\text{-}\overset{\overset{CO_2H}{|}}{\overset{CH_2}{\overset{|}{N}}}\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-NH-}(CH_2)_6\right]_n\text{NH-Chromophore}$$

A metal complex of this ligand, which may also be associated with counterions to provide charge neutrality, is illustrated below:

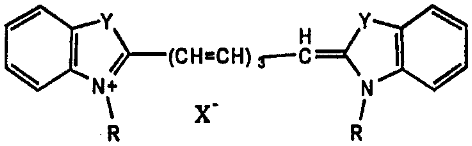


A more specific example of a polymeric ligand in this class and a metal complex of it are illustrated below:



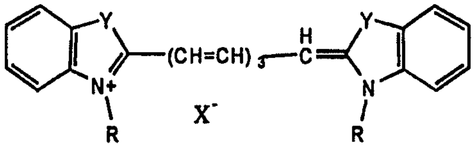


Further examples include:
cyanine dyes, such as heptamethinecyanine dyes, e.g., compounds 4a to 4g Table II on page 26 of Matsuoka (supra)



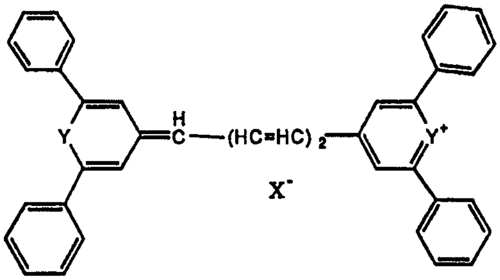
4a: where Y= S,	X=I,	R=Et
4b: where Y= S,	X=ClO_4,	R=Et
4c: where Y= CMe_2,	X=I,	R=Me
4d: where Y=CMe_2,	X=ClO_4,	R=Me
4e: where Y= CH=CH,	X=I,	R=Et
4f: where Y= CH=CH,	X=Br,	R=Et
4g: where Y=CH=CH,	X=ClO_4,	R=Et

and in Table III on page 28 of Matsuoka (supra), i.e.,



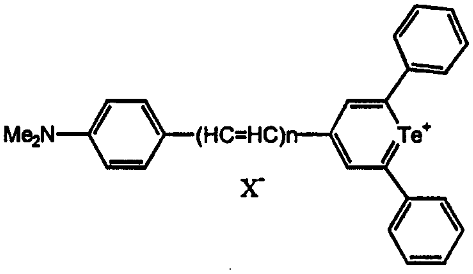
where Y= O, X=I, R=Me
where Y= CMe$_2$, X=I, R=Me
where Y= S, X=Br R=Et;

chalcogenopyrylomethine dyes, e.g., compounds 12 on page 31 of Matsuoka (supra), i.e.,



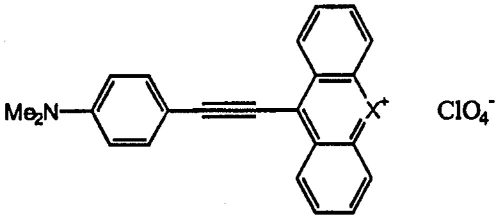
where Y= Te, Se, O or NR;

monochalcogenopyrylomethine dyes, e.g., compounds 13 on page 31, of Matsuoka (supra) i.e.,



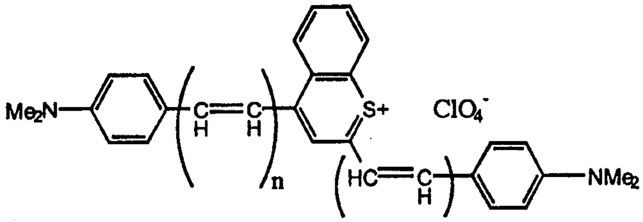
where n = 1 or 2;

pyrilium dyes, e.g., compounds 14 (X= O) on page 32 of Matsuoka (supra), i.e.,



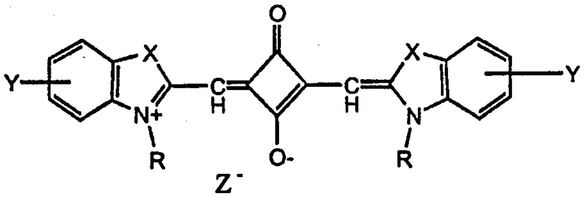
where X = O, S, or Se;

thiapyrilium dyes, e.g., compounds 15 on page 32, and compound I on page 167 of Matsuoka (supra), i.e.,



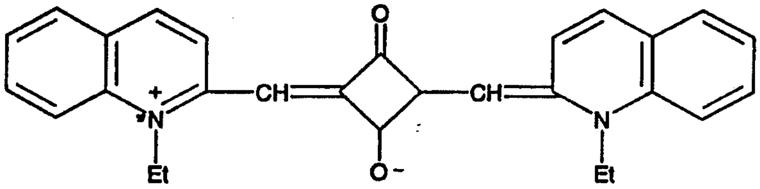
where n = 1 or 2;

squarylium dyes, e.g., compound 10 and Table IV on page 30 of Matsuoka (supra), i.e.,



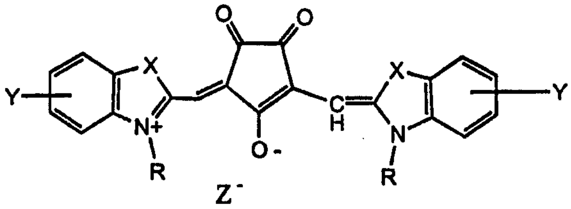
where X = CH=CH, Y = H, and R=Et,
X = S, Y = H, and R=Et, and
X = CMe_2, Y = H, and R=Me,

and compound 6, page 26, of Matsuoka (supra), i.e.,

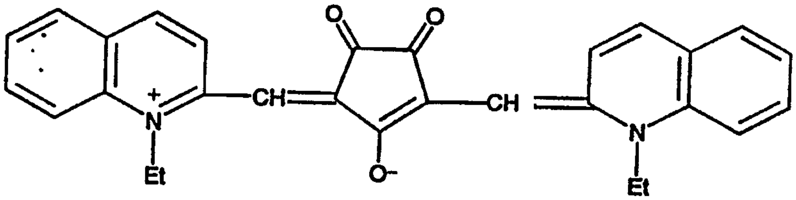


where X = CH=CH, Y = H, and R=Et;

croconium dyes, e.g., compound 9 and Table IV on page 30 of Matsuoka (supra), i.e.,

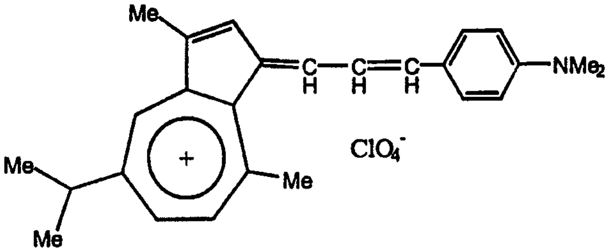


where X = CH=CH, Y = H, and R=Et,
X = S, Y = H, and R=Et,
X = CMe_2, Y = H, and R=Me,
and compound 7, page 26, of Matsuoka (supra), i.e.,

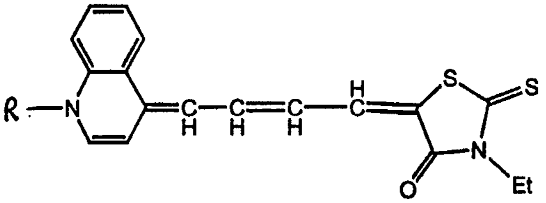


where X = CH=CH, Y = H, and R=Et;

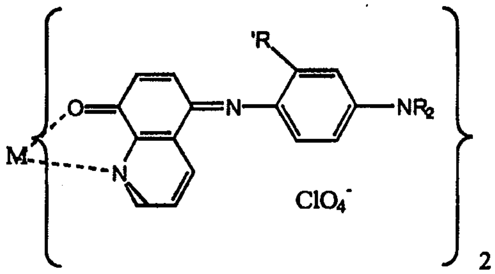
azulenium dyes, e.g., compound 8 on page 27 of Matsuoka (supra), i.e.,



merocyanine dyes, e.g., compound 16, R= Me, on page 32 of Matsuoka (supra), i.e.,

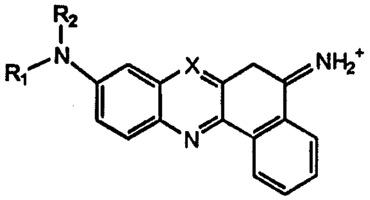


indoaniline dyes such as copper and nickel complexes of indoaniline dyes, e.g., compound 6 on page 63 of Matsuoka (supra), i.e.,



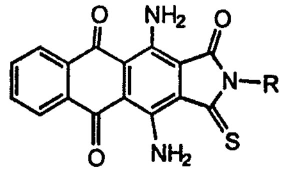
where R = Et, R' = Me, M = Cu,
R = Et, R' = Me, M = Ni,
R = Me, R' = H, M = Cu, or
R = Me, R' = H, M = Ni,

benzo[a]phenoxazinium dyes and benzo[a]phenothiazinium dyes, e.g., as shown on page 201 of Matsuoka (supra), i.e.,

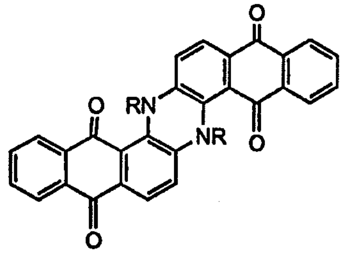


where X = O or S;

1,4-diaminoanthraquinone(N-alkyl)-3'-thioxo-2,3-dicarboximides, e.g., compound 20, on page 41 of Matsuoka (supra)

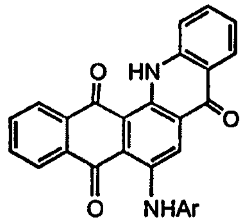


indanthrene pigments, e.g.,

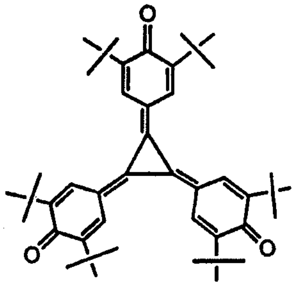


see compound 21 on page 41 of Matsuoka (supra);

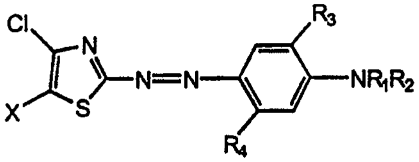
2-arylamino-3,4-phthaloylacridone dyes, e.g., compound 22 on page 41 of Matsuoka (supra)



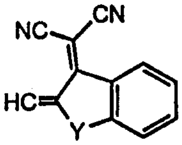
trisphenoquinone dyes, e.g., compound 23 on page 41 of Matsuoka (supra)



azo dyes, e.g., the monoazo dye, compound 2 on page 90 of Matsuoka (supra), i.e.,

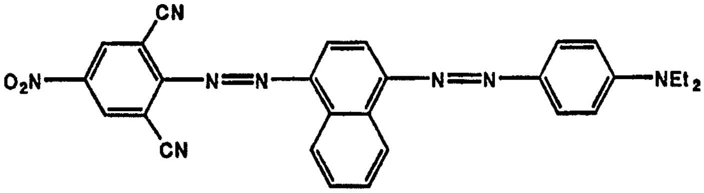


where X = $CH=C(CN)_2$, R_1 = R_2 = Et, R_3 = R_4 = H,
X = $C(CN)=C(CN)_2$, R_1 = R_2 = Et, R_3 = R_4 = H, or
X =

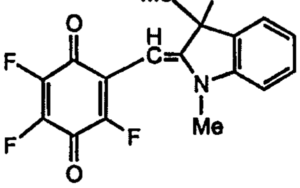


and Y = C=O, R_1 = R_2 = Et, R_3 = R_4 = H,
or Y = SO_2, R_1 = H, R_2 = CH(Me)nBu, R_3 = OMe, and R_4 = NHAc;

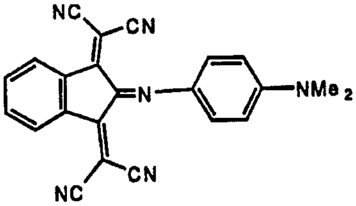
azo dyes, e.g., the polyazo dye, compound 5 on page 91 of Matsuoka (supra), i.e.,



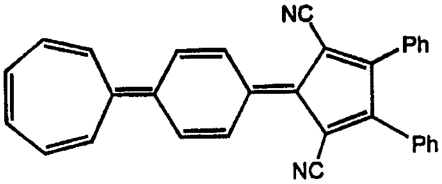
intramolecular charge transfer donor-acceptor infrared dyes, e.g., compounds 6 and 7 on page 91 of Matsuoka (supra), i.e.,



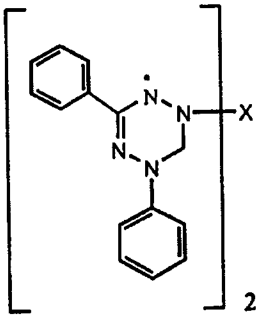
and



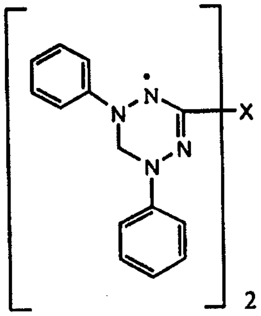
nonbenzenoid aromatic dyes, e.g., compound 8, a tropone, on page 92, of Matsuoka (supra), i.e.,



tetrazine radical dyes, e.g., compound 9 on page 92 of Matsuoka (supra), i.e.,

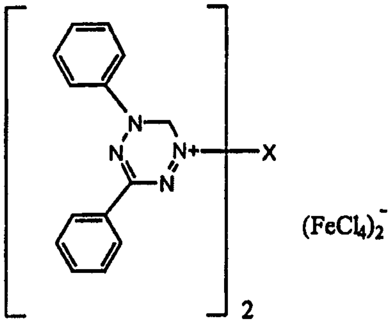


in which, X = p-phenylene or
X = p-terphenylene as well as compound 10 on page 92 of Matsuoka (supra), i.e.,



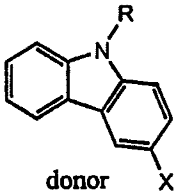
in which X = p-biphenyl;

cationic salts of tetrazine radical dyes, e.g., compound 11 on page 92 of Matsuoka (supra)



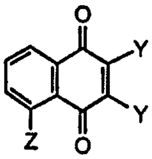
in which X = p-phenylene;

donor-acceptor intermolecular charge transfer dyes, e.g., CT complexes of compounds 13b and 14a to 14c on page 93 of Matsuoka (supra), i.e.,



donor

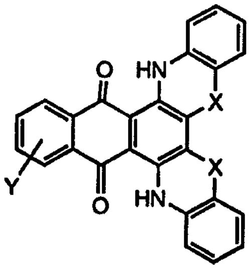
and



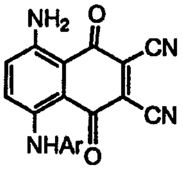
acceptor

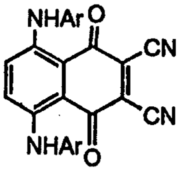
where X = CH=N-N(Ph)$_2$ in the donor and
a) Y = CN, Z = NO$_2$
b) Y = CN, Z = H or
a) Y = Cl, Z = NO$_2$ in the acceptor;

anthraquinone dyes, e.g., compounds 12 (X = S or Se) on page 38 of Matsuoka (supra), i.e.,

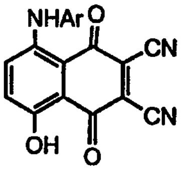


wherein X = S or Se and Y = tetrachloro, tetrabromo, 2,3-dicarboxylic acid, 2,3-dicarboxylic anhydride, or 2,3-dicarboxylic acid N-phenyl imide;
naphthoquinone dyes, e.g., compounds 2, 3, and 4 on page 37, of Matsuoka (supra), i.e.,



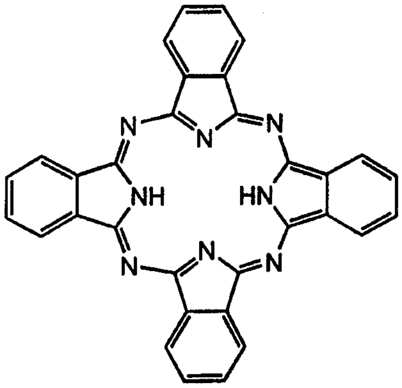


and

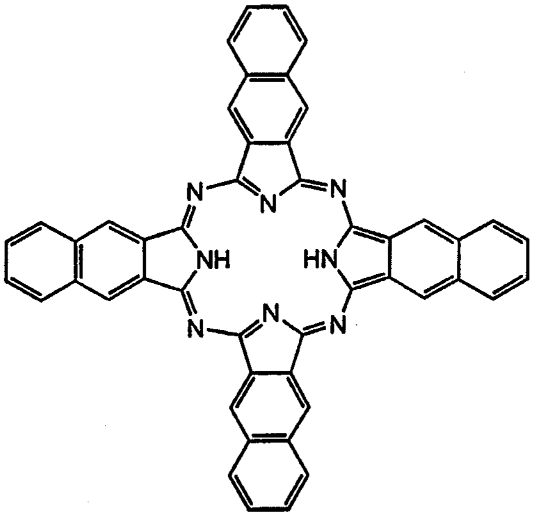


metallated azo dyes such as azo dyes containing nickel, cobalt, copper, iron, and manganese;

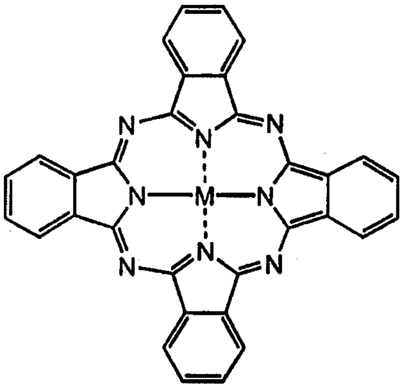
phthalocyanine dyes, e.g., compound 1 in Table II on page 51 of Matsuoka (supra), e.g.,



naphthalocyanine dyes, e.g., compound 3 in Table II on page 51 of Matsuoka (supra), e.g.,

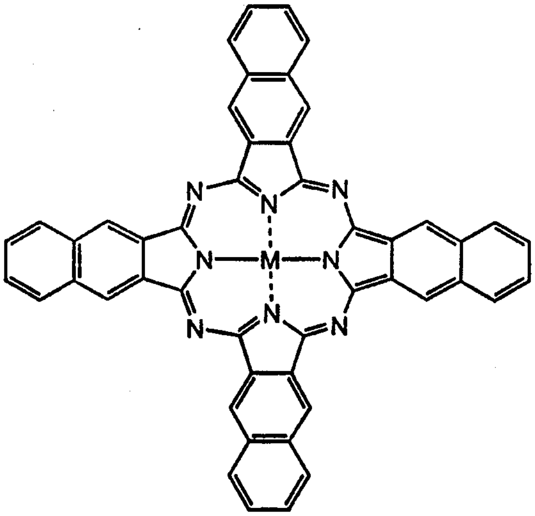


metal phthalocyanines such as phthalocyanines containing aluminum, silicon, nickel, zinc, lead, cadmium, magnesium, vanadium, cobalt, copper, and iron, (especially those containing Al, Si and Zn which are fluorophores) e.g., compound 1 in Table III on page 52 of Matsuoka (supra), e.g.,



in which, for example, M = Mg;

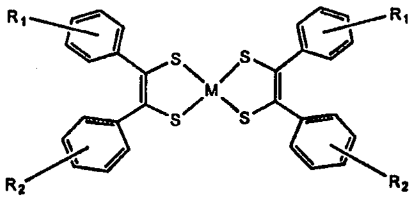
metal naphthalocyanines such as naphthalocyanines containing aluminum, zinc, cobalt, magnesium, cadmium, silicon, nickel, vanadium, lead, copper, and iron (especially those containing Al, Si or Zn which are fluorophores), see compound 3 in Table III on page 52 of Matsuoka (supra), e.g.,



in which, for example, M = Mg;

bis(dithiolene) metal complexes comprising a metal ion such as nickel, cobalt, copper, and iron coordinated to four sulfur atoms in a bis(S,S'-bidentate) ligand complex, e.g., see Table I on page 59 of Matsuoka

(supra)



where

R_1 = R_2 = CF_3, M= Ni,

R_1 = R_2 = phenyl, M= Pd,

R_1 = R_2 = phenyl, M= Pt,

R_1 = C4 to C10 alkyl, R_2 = H, M= Ni,

R_1 = C4 to C10 alkyl, R_2 = H, M= Pd,

R_1 = C4 to C10 alkyl, R_2 = H, M= Pt,

R_1 = R_2 = phenyl, M= Ni,

R_1 = R_2 = p-CH_3-phenyl, M= Ni,

R_1 = R_2 = p-CH_3O-phenyl, M= Ni,

R_1 = R_2 = p-Cl-phenyl, M= Ni,

R_1 = R_2 = p-CF_3-phenyl, M= Ni,

R_1 = R_2 = 3,4,-diCl-phenyl, M= Ni,

R_1 = R_2 = o-Cl-phenyl, M= Ni,

R_1 = R_2 = o-Br-phenyl, M= Ni,

R_1 = R_2 = 3,4,-diCl-phenyl, M= Ni,

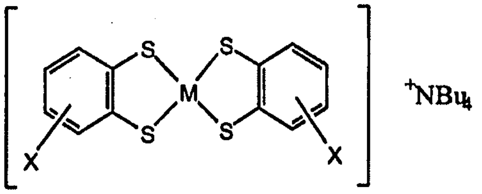
R_1 = R_2 = p-CH_3, M= Ni,

R_1 = R_2 = 2-thienyl, M= Ni,

R_1 = p-$(CH_3)_2$ N-phenyl, R_2 = phenyl, M= Ni, and

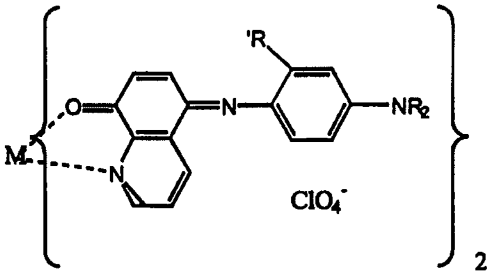
R_1 = p-$(CH_3)_2$ N-phenyl, R_2 =p-H_2N-phenyl, M= Ni;

bis(benzenedithiolate) metal complexes comprising a metal ion such as nickel, cobalt, copper, and iron coordinated to four sulfur atoms in a ligand complex, e.g., see Table III on page 62 of Matsuoka (supra), i.e.,



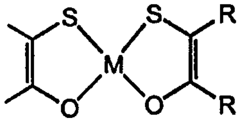
where
X = tetramethyl, M = Ni,
X = 4,5-dimethyl, M = Ni,
X = 4-methyl, M = Ni,
X = tetrachloro, M = Ni,
X = H, M = Ni,
X = 4-methyl, M = Co,
X = 4-methyl, M = Cu, and
X = 4-methyl, M = Fe;

N,O-bidentate indoaniline dyes comprising a metal ion such as nickel, cobalt, copper, and iron coordinated to two nitrogen and two oxygen atoms of two N,O-bidentate indoaniline ligands, e.g., compound 6 in Table IV on page 63 of Matsuoka (supra), e.g.,

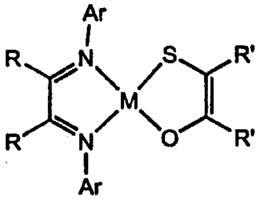


where R = Et, R' = Me, M = Cu,
R = Et, R' = Me, M = Ni,
R = Me, R' = H, M = Cu, and
R = Me, R' = H, M = Ni,

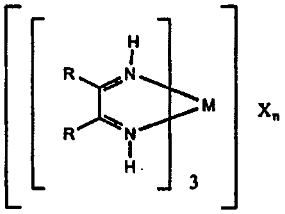
bis(S,O-dithiolene) metal complexes comprising a metal ion such as nickel, cobalt, copper, and iron coordinated to two sulfur atoms and two oxygen atoms in a bis(S,O-bidentate) ligand complex, e.g., see US Patent 3,806,462, e.g.,



α-diimine-dithiolene complexes comprising a metal ion such as nickel, cobalt, copper, and iron coordinated to two sulfur atoms and two imino-nitrogen atoms in a mixed S,S- and N,N-bidentate diligand complex, e.g., see Table II on page 180, second from bottom, of Matsuoka (supra) (also see Japanese patents: 62/39,682, 63/126,889 and 63/ 139,303), e.g.,



and
tris(α-diimine) complexes comprising a metal ion coordinated to six nitrogen atoms in a triligand complex, e.g., see Table II on page 180 of Matsuoka (supra), last compound, (also see Japanese Patents 61/20,002 and 61/73,902), e.g.,



Representative examples of visible dyes include fluorescein derivatives, rhodamine derivatives, coumarins, azo dyes, metallizable dyes, anthraquinone dyes, benzodifuranone dyes, polycyclic aromatic carbonyl dyes, indigoid dyes, polymethine dyes, azacarbocyanine dyes, hemicyanine dyes, barbituates, diazahemicyanine dyes, stryrl dyes, diaryl carbonium dyes, triaryl carbonium dyes, phthalocyanine dyes, quinophthalone dyes, triphenodioxazine dyes, formazan dyes, phenothiazine dyes such as methylene blue, azure A, azure B, and azure C, oxazine dyes, thiazine dyes, naphtholactam dyes, diazahemicyanine dyes, azopyridone dyes, azobenzene dyes, mordant dyes, acid dyes, basic dyes, metallized and premetallized dyes, xanthene dyes, direct dyes, leuco dyes which can be oxidized to produce dyes with hues bathochromically shifted from those of the precursor leuco dyes, and other dyes such as those listed by Waring, D. R. and Hallas, G., in "The Chemistry and Application of Dyes", Topics in Applied Chemistry, Plenum Press, New York, N.Y., 1990. Additional dyes can be found listed in Haugland, R. P., "Handbook of Fluorescent Probes and Research Chemicals", Sixth Edition, Molecular Probes, Inc., Eugene OR, 1996.

Other dye types that can be used in this invention include: Diarylmethane dyes, including but not limited to Acridine dyes, including Acridine Orange, Yellow, Red, Nile Blue, Lucifer Yellow CH, Methylene Blue, Michler's Hydrol Blue, Bindschedler's green, Toluidine Blue, and their derivatives. Triarylmethane dyes, including but not limited to Malachite Green, Crystal Violet, Victoria Blue, Fluorescein, Rose Bengal, Rhodamine, Isosulfan Blue, and their derivatives. Merocyanine dyes, including Merocyanine 540, and their derivatives, Porphyrins.

Other dye types that can be used in this invention include those which at the same time as providing a method of treatment as previously described, can measure some chemical, physiological or biological property of the body to which the dye has been administered.

Naturally, to function in this manner, the dye must be sensitive to the particular chemical, physiological or biological property. Such dyes are preferably used in techniques which incorporate internal imaging of a body, especially the techniques of mammography and tomography.

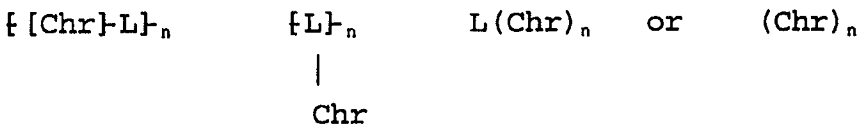
One suitable property that can be measured by dyes used according to the present invention is that of pH. In such instances the dyes can be used both intraoperatively and non-intraoperatively depending on the circumstances. For this aspect of the invention, it is preferable that dyes are chosen which change colour in the pH range 1.5 to 10.0, more preferably 3.0 to 9.0, especially preferably 4.0 to 8.0 and most particularly preferably 6.5 to 8.0. This latter pH range corresponds to that which might actually be encountered in the body. Dyes which emit or absorb light in the visible, infrared or near-infrared regions can be used in this aspect, but preferably such dyes should absorb or emit in the near infrared region of the electromagnetic spectrum.

Examples of commercially available visible dyes that can be used in accordance with the present invention to measure pH include the following (given with colour change and pH range of colour change): nitrazine yellow (yellow to blue, pH 6.0 - 7.0), bromothymol blue (yellow to blue, pH 6.0 - 7.6), brilliant orange (yellow to orange, pH 6.6 - 8.0), phenol red (yellow to red, pH 6.8 - 8.2), m-nitrophenol (colourless to yellow, pH 6.8 to 8.6), cresol red (yellow to red, pH 7.0 to 8.6) and m-cresol purple (yellow to purple, pH 7.4 to 9.0).

Most preferably, the chromophores in the dye compounds of the invention are triphenylmethanes, cyanines, merocyanines, phthalocyanines, naphthalocyanines or porphyrins and the phthalocyanines are used by way of an example of a typical appropriate chromophore in much of the discussion below. However in place of the phthalocyanines discussed, other chromophores of the types listed above, in particular the triphenylmethanes, cyanines, merocyanines, porphyrins and especially naphthalocyanines may be used.

As mentioned above, the dye compounds of the invention may contain a single chromophore ("monomeric" dyes) or they may contain more than one, e.g., 2 to 100, especially 2 to 10, chromophores ("polymeric" dyes). Where the dye compound is polymeric, the structure can be any of the well known polymer structures, e.g., with the chromophores pendant from a molecular (preferably polymeric) backbone, with the chromophores forming part of a polymer backbone, with the chromophores attached to the termini of a branched molecular (preferably polymeric, e.g., dendrimeric) structure or a combination of such structures. In these cases, the organic skeletons of the chromophores will be coupled together, e.g., with pairs of chromophore skeletons being coupled via bonds of linking groups, or several will be coupled to a common backbone structure, which linking groups or backbone structures may be vectors as discussed below. Such structures have been widely described in relation to polychelant compounds useful as MRI contrast agents. However with metal or pseudometal containing chromophores, such as the porphyrins, phthalocyanines, and naphthalocyanines and others mentioned above, a further polymeric structure is possible where linkage of the chromophores is via the metal rather than the organic skeleton. Such polymers are termed cofacial polymers and represent a particular novel aspect of the invention. For the purposes of this text, polychromophores are compounds containing two or more chromophoric groups, which may or may not be chemically different.

Thus such polymeric dye compounds may conveniently have the formulae:



$$\left[[\mathrm{Chr}]-\mathrm{L}\right]_n \qquad \underset{\mathrm{Chr}}{\underset{|}{\left[\mathrm{L}\right]_n}} \qquad \mathrm{L}(\mathrm{Chr})_n \quad \text{or} \quad (\mathrm{Chr})_n$$

where n is an integer with the value 2 or greater (e.g., 2 to 100), Chr is a chromophore and L is a bond, linker group (e.g., a divalent, trivalent or tetravalent organic linker), branched multivalent linker structure (e.g., a dendrimer or starburst polymer), or a vector group (or vector-linker combination) as discussed below.

The linker and vector-linker groups used may be of the types used in the polychelants known from the MRI contrast agent field. Preferably however the linker groups comprise a polyalkyleneoxy component in their linking skeleton, e.g., a component $[(CR_aR_b)_x-(CH_2)_y-O-]_s$, $[(CR_aR_b)_x-(CH_2)_y-]_s$ where x=1, y=1-3, where x=0, y=2-4 and R_a,R_b=H, CH_3, CH_2CH_3 and s is preferably such that the $[(CR_aR_b)_x-(CH_2)_y-O-]_s$, $[(CR_aR_b)_x-(CH_2)_y-]_s$ unit has a molecular weight of 100 to 100000 , especially 1000 to 25000 , most especially 1000 to 5000 . Such units may comprise alkylene units, $(CH_2)_q$, of different lengths, e.g., in blocks such as $((CH_2)_2O)_t((CH_2)_3O)_v$, where t and v are integers having values above 2. Thus for example, the polychromophores may be of formula $Chr-L_1-(PAO-L_2-Chr)_n$ where Chr is a chromophore, optionally and preferably containing a sulphonic acid group, L_1 and L_2 are bonds or linking functions, PAO is a polyalkyleneoxy group and n is an integer having a value of at least 1, e.g., 1 to 100.

In the near-IR polymeric dye compounds according to the invention, it is preferred that each chromophore should carry at least one sulphonic acid (or derivative) group, preferably at least two and more preferably at least three such groups. It is especially preferred that the dye compounds according to the invention contain at least 5, preferably at least 8 sulphonic acid (or derivative groups). For the monomeric dyes, the total number of such groups may for example be as high as 12. For the polymeric dye compounds, the total number will depend upon the degree of polymerization but the total number may for example be as high as ten times the total number of chromophores.

The sulphonic acid groups may be present as the free acid (SO_3H) or as salts thereof with physiologically acceptable anions, e.g., alkali metal, alkaline earth metal, ammonium and organic amine ions (e.g., sodium, potassium, ammonium, ethanolamine, diethanolamine, meglumine, etc.). Alternatively, they can be modified to produce derivatives such as amides.

The presence of, and the nature and degree of derivatization of, the sulphonic acid groups affects the hydrophilicity, lipophilicity and ionic nature of the dye compounds of the invention. This in turn affects their ability to penetrate tumor cells or simply to accumulate around tumor cells. However, both for intraoperative use and for use in post-surgical PDT, penetration of tumor cells by the dye compound is not essential.

In one aspect, a preferred physiologically tolerable contrast agent of this invention comprises at least one chromophoric group attached to a surfactant molecule.

In this invention, a surfactant molecule is defined as an emulsifier or detergent as listed in McCutcheon's Directories, Volume 1: Emulsifiers and Detergents (1994), and which contains at least one chemical functional group selected from the group consisting of an alcohol (OH), a nitrilo group including a primary amine (NH2) and a secondary amine (NH), a carboxylic acid (COOH), a sulfhydryl (SH), a phosphoric acid group, phosphonic acid group, a phenolic group, a sulfonic acid group, a carbon-carbon double bond, and a ketone.

Chemical functional groups in the surfactant molecules can be interconverted by chemical reactions well known to those skilled in the art. For example, a hydroxyl group can be converted to a methanesulfonic acid ester which can be treated with sodium azide and reduced to form an amine group. Carboxylic acid groups and ketones can be reduced to form alcohols, and alcohols can be oxidized to form ketones, aldehydes, and carboxylic acid groups.

Useful surfactant molecules are emulsifiers or detergents which can function as dispersing agents, wetting agents, adsorbents, anticaking agents, soil antiredispositioning agents, antistats, binders, carriers, pearlescents, conditioning agents, hydrotropes, defoamers, emollients, flocculants, humectants, lubricants, opacifiers, plasticizers, preservatives, release agents, scale inhibitors, stabilizers, suspending agents, thickeners, UV absorbers, water repellants, waxes, and polishes, and which contain at least one chemical functional group selected from the group consisting of an alcohol (OH), a nitrilo group including a primary amine (NH2) and a secondary amine (NH), a carboxylic acid (COOH), a sulfhydryl (SH), a phosphoric acid group, a phosphonic acid group, a phenolic group, a sulfonic acid group, a carbon-carbon double bond, and a ketone.

Preferably, the surfactant molecule comprises a polyalkyleneoxide moiety, optionally containing a branching group as defined herein; more preferably a polyalkyleneoxide block copolymeric moiety, optionally containing a branching group as defined herein; and most preferably a polyalkyleneoxide block copolymeric moiety optionally containing a branching group as defined herein and comprising a polypropylene oxide block and a polyethyleneoxide block. Examples of useful surfactant molecules include block copolymers such as AL 2070 available from ICI Surfactants, Antarox block copolymers available from Rhone-Poulenc, Delonic block copolymers available from DeForest, Inc., Hartopol block copolymers available from Texaco Chemical Canada, Macol block copolymers available from PPG Industries, Marlox block copolymers available from Huls America, Pluronic block copolymers including Pluronic F, L, P and R available from BASF Corp., Poly-Tergent block copolymers available from Olin Corp., and Tetronic and Tetronic R block copolymers available from BASF Corp. Currently preferred surfactant molecules include Tetronic and Pluronic block copolymers, and currently most preferred are Tetronic block copolymers.

Chromophoric groups can be attached to surfactant molecules by means of linking groups as defined herein. In one aspect, a chromophoric group can be chemically bound to a surfactant group by means of reaction with a chemical functional group on the surfactant molecule using chemical modifications well known to one skilled in the art. Examples of such reactions include formation of ester linking groups by reaction with an alcohol or phenolic OH group on a surfactant molecule with a chromophoric group containing an acid chloride such as a carboxylic or sulfonic or phosphoric acid chloride or phosphonic acid chloride; formation of amide linking groups by reaction with a primary or secondary amine group on a surfactant molecule with a chromophoric group containing an acid chloride; conversion of a carboxylic acid group or a sulfonic acid group to a reactive intermediate such as an acid chloride or an active ester followed by reaction with a chromophoric group containing an amine or alcohol group.

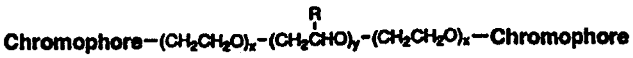
Preferred contrast agents can be prepared from Pluronic and Tetronic block copolymer surfactant molecules by first converting the hydroxyl functional groups to amine groups and then reacting the amine-containing surfactant molecules with chromophoric groups, for example, with chromophoric groups containing isothiocyanate groups, isocyanate groups, and acid chloride groups.

When a surfactant molecule contains more than one chemical functional group, chromophores can be attached to all or to less than all of the chemical functional groups.

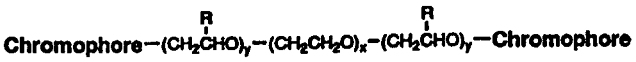
Attachment of chromophores can occur at the ends of the polymer backbone, at the ends of the branching groups, or both. The chromophoric groups can be the same or different. Preferably the chromophoric groups are the same.

In cases where either the surfactant molecule or the attached chromophore are ionically charged, a counterion may be associated to provide charge neutrality. Meglumine is particularly useful for incorporation as a counterion, although other counterions of appropriate charge might also be employed.

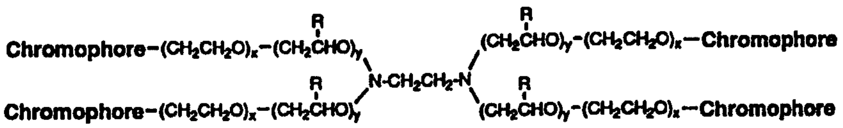
Examples of preferred contrast agents include the following wherein R is a methyl group.

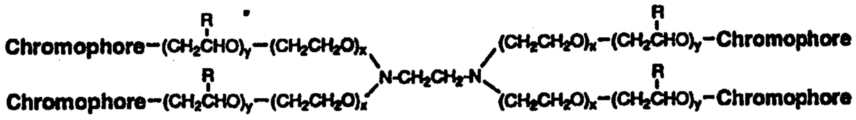


$$\text{Chromophore}-(CH_2CH_2O)_x-(CH_2\overset{R}{C}HO)_y-(CH_2CH_2O)_x-\text{Chromophore}$$



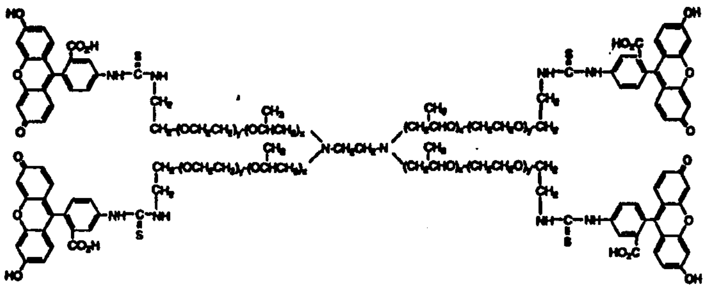
$$\text{Chromophore}-(CH_2\overset{R}{C}HO)_y-(CH_2CH_2O)_x-(CH_2\overset{R}{C}HO)_y-\text{Chromophore}$$





Preferably, the surfactant molecule T-908 (BASF Corporation) can be modified by converting the terminal OH groups to provide an amine group on each branch of the block copolymeric T-908. The modified polymer can then been reacted with an amine-reactive chromophore such as a fluorescein isothiocyanate.

While the product preferably contains four chromophoric groups, it can contain less than four chromophoric groups such as three or two or one. The branches that do not contain a chromophoric group can be terminated by amine groups or by hydroxyl groups. In this example the ratio of x:y is approximately 1:4 and the average molecular weight is approximately 26,000.



Recently, a novel approach to the diagnosis of the spread of cancer from the primary lesion to regional draining lymph nodes has become very popular for both melanoma and breast cancer. That approach is known as Sentinel Lymph Node (SLN) mapping and involves identification of the first node(s) which drain the lesion using either a radiotracer (i.e., ^{99m}TcS) or a low molecular weight blue dye (i.e., Lymphosulfan Blue, 1%) or a combination of both (N. P. Alazraki, et al., Sem. Nucl. Med. Vol 27 (1), 55-67 (1997); R. Pijpers, et al., European J. Nucl. Med. Vol 22 (11), 1238-1241 (1995)) The agent(s) are administered in a peri-lesional location some time before resection of the lesion is performed or in the case of melanoma, they may be administered around the edge of the surgical site after resection of the lesion has already occurred. In either case, staging of the disease is based upon involvement of the first or sentinel lymph node as determined by a number of ex vivo diagnostic tests including standard histology, immunohistochemical staining, and PCR analysis. Thus, if the sentinel node shows no sign of cancer involvement, it is assumed that the sequential lymph nodes are not involved and that the primary cancer has not spread from the lesion. However, if the first node does demonstrate involvement, then the remaining lymph nodes are removed and tested and treatment of the patient is significantly impacted.

The intraoperative dyes proposed in this application may also be used to advantage in SLN. The polymeric dyes will do a better job of being delivered into the draining lymphatics than is the currently used low molecular weight dye and consequently provide for improved contrast during resection of the sentinel node. In addition, the currently used agents are used in a kinetic fashion; that is, their identification of the sentinel node is critically dependent upon both the magnitude of the dose and the time after dosing when the procedure is carried out. Thus, both the radiotracer and the blue dye can and do move beyond the first node if given enough time. The dyes and dye constructs suggested in this application can also be used this way but may also provide true sentinel lymph node agents via delivery to the first node only based upon excess charge, the nature of the charge (i.e., positive vs negative), molecular weight, hydrophobicity/hydrophilicity, and molecular composition. Further, dye + alternative reporter group concepts can be envisioned herein to assist in both the non-invasive location of the sentinel lymph node (i.e., CT X-ray, MRI, ultrasound, nuclear, etc.) followed by the visual assistance during surgical resection of the sentinel node. Such combinations can be soluble constructs or insoluble combinations. For example, polymeric chelators such as those disclosed in US 5,730,968 can be end group modified with chromophores such that they can serve as both MRI and Light imaging contrast agents which can be used as discussed above in SLN. Further, insoluble constructs such as NC 72115 (see working example 19 in WO 96/23524) which comprise both an iodinated, CT dense moiety and a fluorescent moiety can be used as discussed above for SLN.

As discussed with respect to sentinel lymph node imaging, the light imaging agents envisioned within this application can be linked to additional reporting groups for multi-modality imaging (T. Peters, et al., IEEE Transactions on Medical Imaging, vol 15 (2), pp 121-128 (1996), H. Loats, J. Nuclear Medicine vol 34(3), pp 562-566 (1993), M. Staemmler, et al., Endosc. Surg. Allied Technol. Vol 3 (4), pp 199-203 (1995), C. Barillot, et al., Eur. J. Radiol. Vol 17, pp22-27 (1993)) within target tissues in addition to lymph nodes. For example, a combined MRI and light contrast agent can be used to provide both diagnostic imaging and intraoperative assistance during surgery. Polymeric agents such as the polymeric chelators discussed in US 5,730,968 modified with end group chromophores can be used for passive targeting to solid tumors or sites of inflammation for both diagnostic imaging and surgical assistance. An area wherein multi-modal imaging may be especially useful is in the diagnosis and subsequent arthroscopic surgery of joint injuries, gall bladder removal, coronary bipass, etc., as more and more surgeries are being converted to arthoscopic or endoscopic procedures. Thus, the non-light portion of the agent woudl be useful in diagnosing the situation while the same agent would aid in locating the lesion, site of disease by the fibre optic of the arthroscope. These agents could be soluble or insoluble as cited for sentinel lymph node imaging (i.e., CT X-ray plus fluorescent dye, working example 19 in WO 96/23524) where the insoluble agents may be formulated as liposomes, emulsions, or nanoparticles which are especially useful at imaging tissues which are rich in cells of the mononuclear phagocytic system (i.e., Liver, spleen, lungs, bone marrow, etc.).

A particularly exciting application for such a single agent which comprises a multi-modal benefit is in the emerging technology of fusion imaging. In situations wherein the identity of a lesion is problematic, the utility of double imaging modalities followed by "fusion" of the images into a single display can delineate the area of disease. While this technology is critically dependent upon signficant computer power, the utility of contrast agents for such a technology is also indicated. For example, one can envision an agent which comprises an MRI sensitive moiety and a fluorescent moiety which is quenched by molecular oxygen. Imaging of a skin lesion would involve both MRI and fluorescent imaging such that the entire lesion would image by MRI but only the necrotic areas would fluoresce. Thus, fusion of the images would clearly detail not only the anatomy of the lesion but the functionality of the lesion as well. An interesting example of such a potential agent is given in working example 16 of this application.

As mentioned above, the dye compounds may passively target the tumor tissue or cells or they may be attached to an active targeting moiety, a "vector", which serves to modify their biodistribution pattern, e.g., to affect blood residence times or affect excretion and so allow accumulation at tumor sites over several passes or allow greater penetration of the dye compound through the vasculature or to bind the dye to receptors at tumor cell surfaces or to other receptors which are more abundant in regions of tumor growth. Such vector bound dye compounds are considered to fall within the definition of the compounds of the invention. Appropriate vectors include antibodies, antibody fragments, receptor-binding peptides and peptoids, tumor-targeting drug compounds, blood residence prolonging compounds (e.g., polyalkylene oxides such as PEG), folic acid and derivatives thereof, and the like. Transferrin is a particularly suitable vector for targetting a chromophore to a growing tumor due to the general up-regulation of transferrin receptors at growing tumors.

Vectors and their attachment, for example bifunctional linker compounds, have been widely described in relation to contrast agents for MRI and scintigraphy and in relation to targeted radiopharmaceuticals in general. See for example "Handbook of targeted delivery of imaging agents", Ed. V.P. Torchilin, CRC, Boca Raton, USA, 1995. Targeting can likewise be achieved by loading the dye compound into vesicles (e.g., liposomes, micelles, micro balloons etc) and optionally using ultrasound or laser light to reduce the integrity of the vesicle membrane (e.g., by the "pop-corn" effect of high intensity ultrasound (see US-A-5425366) or gas-containing vesicles or by the heating effect on the vesicle contents of laser illumination at the chromophore's absorption wavelength) and to release the dye compound at the desired location in the body. In this way, the dosage of dye compound needed may be significantly reduced relative to that needed with passive delivery. Vesicle production for contrast agent (or drug) delivery is well described in relation to contrast agents used in ultrasound, MRI and X-ray (e.g., CT-imaging), e.g., in the patent publications of Nycomed Imaging, Nycomed Salutar and Unger.

As mentioned, an alternative to the passive targeting of dyes to tumors or to specific body tissues, the dyes can be actively directed to desired sites in the body by the covalent attachment of appropriate targeting molecules, sometimes referred to as vectors.

These vectors are typically macromolecular and may be directly linked to the dye or, in those cases where this interferes with the targeting capability of the vector, an intermediate molecule, or linker, may be used.
Thus the vector-targeted dye compounds of the invention may be considered to be of formula V-L-R, where R is the chromophore or polychromophore (i.e., the residue of a dye), V is a vector moiety which can bind to sites (receptors) on cells of a tissue of interest, and L is a bond or a linking group which joins the vector moiety to the chromophore.

The term "residue" is used herein in context with a chemical entity. Said chemical entity comprises, for example, a vector moiety, or a dye, or a linking group, or a vector reactive group, or an receptor recognizing group, or an immunoreactive material, or an immunoreactive protein, or an antibody, or an antibody fragment, or a protein, or a peptide, or a small organic molecule, or a cross-linking agent such as a heterobifunctional cross-linking agent, or a spacing group. The term "residue" is defined as that portion of said chemical entity which exclusively remains when one or more chemical bonds of which said chemical entity is otherwise comprised when considered as an independent chemical entity, are altered, modified, or replaced to comprise one or more covalent bonds to one or more other chemical entities. Thus, for example, in one aspect of the V-L-R composition of matter, the residue of a dye is comprised of a dye which is at least monovalently modified through attachment to the residue of another chemical entity such as, for example, to the residue of a linking group.

As used herein, the terms "receptor" and "antigen" refer to a chemical group in a molecule which comprises an active site in said molecule, or to an array of chemical groups in a molecule which comprise one or more active sites in said molecule, or to a molecule comprised of one or more chemical groups or one or more arrays of chemical groups, which group or groups or array of groups comprise one or more active sites in said molecule. An "active site" of a receptor has a specific capacity to bind to or has an affinity for binding to a vector. With respect to use with the term "receptor" or with the term "active site in a receptor", the term "vector" as used herein refers to a molecule comprised of a specific chemical group or a specific array of chemical groups (receptor recognizing group) which molecule, group, or array of groups is complementary to or has a specific affinity for binding to a receptor, especially to an active site in a receptor, or which otherwise modifies the biodistribution of the overall composition of matter in a desired manner. Examples include cell surface antigens, cell surface and intracellular receptors which bind hormones; and cell surface and intracellular receptors which bind drugs. The sites of specific association of specific binding of hormones to said cellular receptors; and of specific binding of drugs to cellular receptors are examples of active sites of said receptors, and the hormones ,or the drugs are examples of vectors for the respective receptors.

The vector group, V, can be selected from a wide variety of naturally occurring or synthetically prepared materials, including, but not limited to enzymes, amino acids, peptides, polypeptides, proteins, lipoproteins, glycoproteins, lipids, phospholipids, hormones, growth factors, steroids, vitamins, polysaccharides, lectins, toxins, nucleic acids (including oligonucleotides and peptidic nucleic acids), haptens, avidin and derivatives thereof, biotin and derivatives thereof, antibodies (monoclonal and polyclonal), anti-antibodies, antibody fragments and antigenic materials (including proteins and carbohydrates). The vector group, V, can be also be selected from, but not limited to, components or products of viruses, bacteria, protozoa, fungi, parasites, rickettsia, molds, as well as animal and human blood, plant source material, tissue and organ components. Furthermore, the vector group, V, can be a pharmaceutical drug or synthetic analog of any of the materials mentioned above as well as others known to one skilled in the art. Additional specific vector groups are described in WO 96/40285 which is incorporated herein in its entirety.

The vector, V, preferably is an antibody, antibody fragment, protein or peptide which recognizes and is specific for a tumor associated antigen or receptor. In some embodiments, V can contain a receptor recognizing group covalently bonded thereto through a chemical bond or a linking group derived from the residue of a receptor recognizing group and the residue of a reactive group on V. As used herein, the term "receptor recognizing group" which can be abbreviated by "RRG" also includes an organic compound which is capable of covalently bonding the vector and which is found in a living organism or is useful in the diagnosis, treatment or genetic engineering of cellular material or living organisms, and which has a capacity for interaction with another component ("active site" of a receptor) which may be found in biological fluids or associated with cells to be treated such as tumor cells.

The RRG can be selected from the same wide variety of naturally occurring or synthetically prepared materials mentioned above. In addition, an RRG can be any substance which when presented to an immunocompetent host will result in the production of a specific antibody capable of binding with that substance, or the antibody so produced, which participates in an antigen-antibody reaction.

Preferred vectors are antibodies and various immunoreactive fragments thereof, proteins and peptides as long as they contain at least one reactive site for reaction with a vector reactive group or with linking groups (L) as described herein. That site can be inherent to the vector or it can be introduced through appropriate chemical modification of the vector. In addition to antibodies and fragments produced by the techniques outlined herein, other antibodies, proteins, and peptides produced by the techniques of molecular biology, phage display, and genetic engineering are specifically included.

As used herein, the term "antibody fragment" refers to a vector which comprises a residue of an antibody, which antibody characteristically exhibits an affinity for binding to an antigen. The term affinity for binding to an antigen, as used herein, refers to the thermodynamic expression of the strength of interaction or binding between an antibody combining site and an antigenic determinant and, thus, of the stereochemical compatibility between them; as such, it is the expression of the equilibrium or association constant for the antibody-antigen interaction. The term affinity, as used herein, also refers to the thermodynamic expression of the strength of interaction or binding between a ligand and a receptor and, thus, of the stereochemical compatibility between them; as such, it is the expression of the equilibrium or association constant for the ligand/receptor interaction.

Antibody fragments exhibit at least a percentage of said affinity for binding to said antigen, said percentage being in the range of 0.001 per cent to 1,000 per cent, preferably 0.01 per cent to 1,000 per cent, more preferably 0.1 per cent to 1,000 per cent, and most preferably 1.0 per cent to 1,000 per cent, of the relative affinity of said antibody for binding to said antigen.

An antibody fragment can be produced from an antibody by a chemical reaction comprising one or more chemical bond cleaving reactions; by a chemical reaction comprising one or more chemical bond forming reactions employing as reactants one or more chemical components selected from a group comprised of amino acids, peptides, carbohydrates, linking groups as defined herein, spacing groups as defined herein, vector reactive groups as defined herein, and antibody fragments such as are produced as described herein and by a molecular biological process, a bacterial process, or by a process comprised of and resulting from the genetic engineering of antibody genes.

An antibody fragment can be derived from an antibody by a chemical reaction comprised of one or more of the following reactions:

(a) cleavage of one or more chemical bonds of which an antibody is comprised, said bonds being selected from, for example, carbon-nitrogen bonds, sulfur-sulfur bonds, carbon-carbon bonds, carbon-sulfur bonds, and carbon-oxygen bonds, and wherein the method of said cleavage is selected from:

(i) a catalysed chemical reaction comprising the action of a biochemical catalyst such as an enzyme such as papain or pepsin which to those skilled in the art are known to produce antibody fragments commonly referred to as Fab and Fab'2, respectively;

(ii) a catalysed chemical reaction comprising the action of an electrophilic chemical catalyst such as a hydronium ion which, for example, favorably occurs at a pH equal to or less than 7;

(iii) a catalysed chemical reaction comprising the action of a nucleophilic catalyst such as a hydroxide ion which, for example, favorably occurs at a pH equal to or greater than 7; and

(iv) a chemical reaction comprised of a substitution reaction employing a reagent which is consumed in a stoichiometric manner such as substitution reaction at a sulfur atom of a disulfide bond by a reagent containing a sulfhydryl group;

(v) a chemical reaction comprised of a reduction reaction such as the reduction of a disulfide bond; and

(vi) a chemical reaction comprised of an oxidation reaction such as the oxidation of a hydroxyl group or the oxidation of a carbon-carbon bond of a vicinal diol group such as occurs in a carbohydrate moiety; or

(b) formation of one or more chemical bonds between one or more reactants, such as formation of one or more covalent bonds selected from, for example, carbon-nitrogen bonds (such as, for example, amide bonds, amine bonds, hydrazone bonds, and thiourea bonds), sulfur-sulfur bonds such as disulfide bonds, carbon-carbon bonds, carbon-sulfur bonds, and carbon-oxygen bonds, and employing as reactants in said chemical bond formation one or more reagents comprised of amino acids, peptides, carbohydrates, linking groups as defined herein, spacing groups as defined herein, vector reactive groups as defined herein, and antibody fragments such as are produced as described in (a), above; or

(c) an antibody fragment can be derived by formation of one or more non-covalent bonds between one or more reactants. Such non-covalent bonds are comprised of hydrophobic interactions such as occur in an aqueous medium between chemical species that are independently comprised of mutually accessible regions of low polarity such as regions comprised of aliphatic and carbocyclic groups, and of hydrogen bond interactions such as occur in the binding of an oligonucleotide with a complementary oligonucleotide; or

(d) an antibody fragment can be produced as a result of the methods of molecular biology or by genetic engineering of antibody genes, for example, in the genetic engineering of a single chain immunoreactive group, a Fv fragment or a minimal recognition unit.

An antibody fragment can be produced as a result of a combination of one or more of the above methods.

If desired, a vector can be modified or chemically altered to provide reactive groups for attaching to the residues of a receptor moiety or antigen found in or on tissues and cells of interest. Such techniques include the use of linking moieties and chemical modification such as described in WO-A-89/02931 and WO-A-89/2932, which are directed to modification of oligonucleotides, and U.S. Patent No. 4,719,182.

A preferred use for the compositions of this invention are for the intraoperative delineation of tumors. Preferred vectors therefore include antibodies (sometimes hereinafter referred to as Ab) to tumor-associated antigens. Specific non-limiting examples include B72.3 and related antibodies (described in U.S. Patent Nos. 4,522,918 and 4,612,282) which recognize colorectal tumors; 9.2.27 and related anti-melanoma antibodies; D612 and related antibodies which recognize colorectal tumors; UJ13A and related antibodies which recognize small cell lung carcinomas; NRLU-10, NRCO-02 and related antibodies which recognize small cell lung carcinomas and colorectal tumors (Pan-carcinoma); 7E11C5 and related antibodies which recognize prostate tumors; CC49 and related antibodies which recognize colorectal tumors; TNT and related antibodies which recognize necrotic tissue; PR1A3 and related antibodies which recognize colon carcinoma; ING-1 and related antibodies, which are described in WO-A-90/02569; B174, C174 and related antibodies which recognize squamous cell carcinomas; B43 and related antibodies which are reactive with certain lymphomas and leukemias; and anti-HLB and related monoclonal antibodies; and other tumor, tissue or cell-specific antibodies known to those skilled in the art.

More preferred vectors are proteins, especially recombinant human proteins, such as are produced or modified by molecular biological, phage display or genetic engineering techniques, which modifications comprise the independent incorporation, substitution, insertion, and deletion of specific amino acids in a peptide sequence of said protein to produce recombinant human proteins containing an RRG which has an affinity for binding to an active site of a receptor. A thus-modified recombinant protein vector has an affinity for an active site of a receptor which is greater than the affinity of the natural, unmodified, vector for the active site of the receptor

In another embodiment, the vector (or Vector-Linker component of V-L-R) comprises a fusion protein. As used herein, the term "fusion protein" refers to a genetically engineered material comprised of a protein whose coding region is comprised of the coding region of a residue of a first protein fused, in frame, to the coding region of a residue of a second protein. Preferably, said fusion protein is comprised of a protein whose coding region is comprised of the coding region of a residue of an RRG fused, in frame, to the coding region of one or more residues of a vector or a linker which linker can be a protein or peptide. The above genetically engineered fusion protein comprising the vector can be comprised of a protein whose coding region is independently comprised of the coding region of a residue of a human or of a non-human first protein fused, in frame, to the coding region of a residue of a human or non-human second protein. Preferably, said coding regions are independently human and bacterial or modified by genetic engineering techniques as above.

Even more preferred vectors are peptides, oligopeptides or peptoids, which vectors are composed of one or more amino acids whose sequence and composition comprised a molecule, specific chemical group or a specific array of chemical groups, which are complementary to or have a specific affinity for binding to a receptor, especially to an active site in a receptor. Such peptides may be compositionally identical to the amino acids that comprise the RRG of antibodies, antibody fragments, proteins or fusion proteins that recognize the same receptor. Alternatively, such peptidic vectors may not have an amino acid sequence identical to other RRGs but will be structurally or three-dimensionally equivalent to other RRGs that bind the same receptor. Such equivalent peptides may be identified by molecular biological techniques such as point mutation, phage display, genetic engineering and others techniques known to those skilled in the art. In addition, peptidic or oligopeptidic vectors may be designed *de novo* using the well-practised methodologies of computational chemistry and peptide synthesis. Synthetic peptidic vectors are not restricted to linear arrays of amino acids but may also be cyclic and contain more than one RRG per peptide. Peptide or peptoid vectors may be composed of L-amino acids, D-amino acids, non-naturally occuring amino acids, synthetic amino acid substitutes, organic molecules or mixtures of all of these, and bonded to each other by peptide (amide) or non-amide bonds.

Especially preferred vectors are peptidomimetic molecules, which molecules are fully synthetic organic materials that are the stuctural or functional equivalent of RRGs derived or identified from antibodies, antibody fragments, proteins, fusion proteins, peptides or peptoids, and that have affinity for the same receptor. Other vectors which are considered to be peptidomimetic include chemical entities, such as drugs, for example, which show affinity for the receptor, and especially the active site of the receptor, of interest. Peptidomimetic vectors may be identified through the use of molecular biological techniques such as point mutation, phage display, genetic engineering and others techniques know to those skilled in the art. Peptidomimetic vectors may be designed and synthesized *de novo* using current chemical methods as well as the techniques of computational chemistry and combinatorial chemistry.

As used herein, "Vector reactive group" refers to one or more chemical groups which can react with a reactive functional group typically found on or introduced into a vector, to form a bond between the linking group and the vector. As used herein, the term "linking group" is a chemical moiety that connects together at least two molecules, at least the residue of one molecule with another molecule, or at least the residue of one molecule with the residue of another molecule. It is specifically contemplated that a vector reactive group can be used to conjugate a dye-linker of this invention to a non-protein biomolecule as well as to a non-biological molecule such as a synthetic chemical substance, for example, a drug or other molecule that has an affinity for the active site of a receptor that is of interest. Vector reactive groups can also be used for the purposes of detection of such a molecule in a mixture which may contain such a synthetic chemical substance and which substance contains a group that is reactive with the vector reactive group. Thus, the vector reactive groups useful in the practice of this invention include those groups which can react with any molecule, preferably a biological molecule (such as a protein, a carbohydrate, a nucleic acid, and a lipid) containing a reactive group to form a linking group between the dye and the molecule. If the molecule is a protein, preferred reactive groups include amine groups and sulfhydryl groups. Especially preferred biological molecules contain a RRG as described above.

The vector reactive groups useful in the practice of this invention also include those groups which can react with any biological molecule that is chemically modified, for example, by oxidation, by reduction, or by covalent bond formation such as by amide bond formation with another chemical species such as, for example, an amine, an amino acid, a substituted amine, or a substituted amino acid, to introduce a reactive group into the biological molecule, to form a linking group between the dye and the chemically modified biological molecule.

Preferred vector reactive groups can be selected from, but are not limited to, groups that will react directly with an amine group such as a lysine epsilon amine group or a terminal amine group in a protein or peptide or with a sulfhydryl group such as a cysteine sulfhydryl group commonly found on a protein or other biological molecule. Examples of such protein reactive groups include active halogen-containing groups such as chloromethylphenyl groups, chloromethylcarbonyl groups, and iodomethylcarbonyl groups; activated 2- (leaving group) -substituted ethylsulfonyl and ethylcarbonyl groups such as 2-chloroethylsulfonyl groups and 2-chloroethylcarbonyl groups; vinylsulfonyl groups; vinylcarbonyl groups; oxiranyl groups; isocyanato groups; isothiocyanato groups; aldehydo groups; aziridyl groups; succinimidoxycarbonyl groups; activated acyl groups such as carboxylic acid halide groups; anhydride groups; thioester groups; carbonates such as nitrophenylcarbonates; sulfonic acid esters and chlorides; phosphoramidates; cyanuric monochlorides and cyanuric dichlorides; and other groups known to be useful in conventional photographic gelatin hardening agents.

The above listed vector reactive groups can react with a protein or vector which is chemically modified to contain reactive amine groups and sulfhydryl groups. Amine groups can be introduced by well known techniques such as, for example, nitration of an aromatic group followed by reduction, by conversion of a primary amide to an amine by reduction, by conversion of a hydroxyl group of an alcohol into a sulfonic acid ester followed by displacement with an azide group and subsequent reduction to an amine, and the like. Sulfhydryl groups can be introduced by well known techniques such as, for example, by conversion of a hydroxyl group of an alcohol into a sulfonic acid ester followed by displacement with sodium hydrosulfide, by dehydrative amide bond formation between an amine group of a protein and a carboxylic acid group of an acetylated cysteine using a carbodiimide reagent, and the like.

In addition, when a protein, peptide, peptoid or peptidomimetic can be chemically modified such as by partial oxidation to introduce an aldehyde group or a carboxylic acid group, a preferred vector reactive group can be selected from amino, aminoalkyl, aminoaryl, alkylamino, arylamino, hydrazino, alkylhydrazino, arylhydrazino, carbazido, semicarbazido, thiocarbazido, thiosemicarbazido, sulfhydryl, sulfhydrylalkyl, sulfhydrylaryl, hydroxy, carboxy, carboxyalkyl and carboxyaryl. The alkyl portions of the protein reactive group can contain from 1 to about 20 carbon atoms, and the aryl portions of the protein reactive group can contain from about 6 to about 24 carbon atoms.

An additional preferred vector reactive group can comprise a residue of a crosslinking agent. A useful crosslinking agent can react with a functional group such as, for example, an amine or sulfhydryl or carboxylic acid group or aldehyde group found in a linker and with a functional group such as, for example, an amine or sulfhydryl or carboxylic acid group or aldehyde group found in a vector or in a chemically modified protein or biological molecule such as described above. The residues of certain useful crosslinking agents, such as, for example, difunctional gelatin hardeners, bisepoxides and bisisocyanates become a part of, i.e., a linking group in, a V-L-R conjugate which is formed as a result of the crosslinking reaction of such a crosslinking vector reactive group with a dye. Vector reactive groups derived from various heterobifunctional cross-linking reagents such as those listed in the Pierce Chemical Company Catalog and handbook - Protein Modification Section, (1994/5) are useful and non-limiting examples of such reagents include:

Sulfo-SMCC: Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate
Sulfo-SIAB: Sulfosuccinimidyl (4-iodoacetyl)aminobenzoate.
Sulfo-SMPB: Sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate.
2-IT: 2-Iminothiolane.
SATA: N-Succinimidyl S-acetylthioacetate.

Other useful crosslinking agents, however, facilitate the crosslinking, for example, as consumable catalysts, and are not present in the final conjugate. Examples of such crosslinking agents are carbodiimide and carbamoylonium crosslinking agents as disclosed in U.S. Patent 4,421,847, the disclosure of which is hereby incorporated herein by reference in its entirety, and the dication ethers of U.S. Patent 4,877,724, the disclosure of which is hereby incorporated herein by reference in its entirety. With these crosslinking agents, one of the reactants must have a carboxyl group and the other an amine or sulfhydryl group. The crosslinking agent first reacts selectively with the carboxyl group, preferably a carboxyl group on a vector, then is split out during reaction of the "activated" carboxyl group with an amine, preferably an amine group of a linker, to form an amide bond between the vector and a dye of this invention, thus covalently bonding the two moieties. An advantage of this approach is that crosslinking of like molecules, e.g., dye with dye, can be avoided, whereas the reaction of bifunctional crosslinking agents is nonselective so that unwanted crosslinked molecules can be obtained.

Additional preferred vector reactive groups include semicarbazido; thiocarbazido; thiosemicarbazido; isocyanato and isothiocyanato; vinyl sulfonylalkyloxy, the alkylene group of which preferably contains from 2 to 10 carbon atoms; vinyl sulfonylalkylpoly(oxyalkyl)oxy, the alkylene group of the sulfonylalkyl portion of which preferably contains from 2 to 10 carbon atoms; the alkylene group of the polyoxyalkyl portion preferably contains from 2 to 10 carbon atoms, such poly(oxyalkyl) portion preferably comprising a poly(oxyethylene) group or a poly(oxyethylene)-co-poly(oxypropylene) copolymer group, and the polymer contains from 2 to about 100 monomeric oxyalkylene units; amidoalkyloxy, the alkylene group of which preferably contains from 1 to 10 carbon atoms; hydrazidoalkyloxy, the alkylene group of which preferably contains from 1 to 10 carbon atoms; azidocarbonylalkyloxy, the alkylene group of which preferably contains from 1 to 10 carbon atoms; aryloxycarbonyloxyalkyloxy, the alkylene group of which preferably contains from 2 to 10 carbon atoms, and the aryl group of which is as described above; aryloxycarbonyl(polyoxyalkyl)oxy, the aryl group of which is as described above, and the alkylene group of the polyoxyalkyl portion preferably contains from 2 to 10 carbon atoms and is as described above, such poly(oxyalkyl) portion preferably comprising a poly(oxyethylene) group or a poly(oxyethylene)-co-poly(oxypropylene) copolymer group, and the polymer contains from 2 to about 100 monomeric oxyalkylene units; triazines such as 4,6-dichloro-2-triazinylamino, 4,6-dichloro-2-triazinyloxy, 4,6-dichlorotriazinyl-2-oxy(polyalkyloxy), 4-alkoxy-6-chloro-2-triazinyloxy, and

4-alkoxy-6-chloro-2-triazinyl(polyoxyalkyl)oxy, the alkyl groups of the alkoxy portions preferably each containing from 2 to 10 carbon atoms, and the alkylene groups of the polyoxyalkyl portions preferably each containing from 2 to 10 carbon atoms, such a poly(oxyalkyl) portion preferably comprising a poly(oxyethylene) group or a poly(oxyethylene)-co-poly(oxypropylene) copolymer group, in which the polymer contains from 2 to about 100 monomeric oxyalkylene units; formylalkyl, the alkyl group of which preferably contains from 1 to 10 carbon atoms; aminoalkyl, the alkyl group of which preferably contains from 1 to 10 carbon atoms;

active esters, for example, succinimidoxycarbonyl;

active anhydrides and mixed anhydrides;

active carbonates such as arylcarbonatoaryl, alkylcarbonatoaryl, arylcarbonatoalkyl, and alkylcarbonatoalkyl, the alkyl groups of which preferably contain from 2 to 10 carbon atoms and are as described above, and the aryl groups of which are preferably comprised of a six membered ring containing electron withdrawing substituents such as, for example, nitro and halogen, and optionally containing water solubilizing groups such as a sulfonate salt;

sulfhydryl;

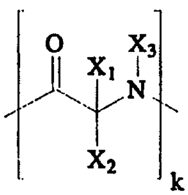
sulfhydrylalkyl, the alkyl group of which preferably contains from 1 to 10 carbon atoms;

thioalkylcarbonylaminoalkyloxy, the alkylene group of the thioalkylcarbonyl portion preferably containing from 1 to 10 carbon atoms, and the alkylene group of the aminoalkyloxy portion preferably containing from 2 to 10 carbon atoms;

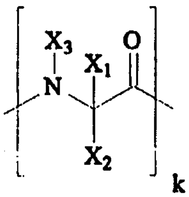
maleimidoalkylcarbonylaminoalkyloxy, the alkylene group of the maleimidoalkylcarbonyl portion preferably containing from 1 to 10 carbon atoms, and the alkylene group of the aminoalkyloxy portion preferably containing from 2 to 10 carbon atoms;

azido;
iodoalkylcarbonylamino, the alkylene group of which contains from 1 to 10 carbon atoms;
amidatoalkylamino, the alkylene group of which contains from 1 to 10 carbon atoms; and
amidatoarylalkylamino, the alkylene group of which contains from 1 to 10 carbon atoms, and the aryl group of which is as described above.

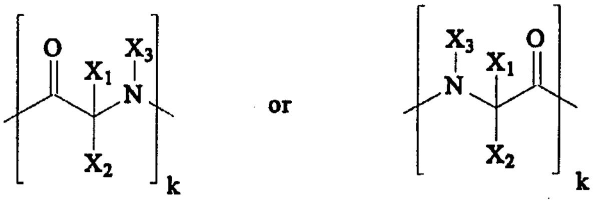
The residues of the vector are independently linked to the other components of this invention through a chemical bond or a linking group. Preferred linking groups may be derived from vector reactive groups and so include nitrogen atoms in groups such as amino, imido, nitrilo and imino groups; alkylene, preferably containing from 1 to 18 carbon atoms such as methylene, ethylene, propylene, butylene and hexylene, such alkylene optionally being interrupted by 1 or more heteroatoms such as oxygen, nitrogen and sulfur or heteroatom-containing groups; carbonyl; sulfonyl; sulfinyl; ether; thioether; ester, i.e., carbonyloxy and oxycarbonyl; thioester, i.e., carbonylthio, thiocarbonyl, thiocarbonyloxy, and oxythiocarboxy; amide, i.e., iminocarbonyl and carbonylimino; thioamide, i.e., iminothiocarbonyl and thiocarbonylimino; thio; dithio; phosphate; phosphonate; urelene; thiourelene; urethane, i.e., iminocarbonyloxy, and oxycarbonylimino; an amino acid linkage, i.e., a



or



group wherein k=1 and X_1, X_2, X_3 independently are H, alkyl, containing from 1 to 18, preferably 1 to 6 carbon atoms, such as methyl, ethyl and propyl, such alkyl optionally being interrupted by 1 or more heteroatoms such as oxygen, nitrogen and sulfur, substituted or unsubstituted aryl, containing from 6 to 18, preferably 6 to 10 carbon atoms such as phenyl, hydroxyiodophenyl, hydroxyphenyl, fluorophenyl and naphthyl, aralkyl, preferably containing from 7 to 12 carbon atoms, such as benzyl, heterocyclyl, preferably containing from 5 to 7 nuclear carbon and one or more heteroatoms such as S, N, P or O, examples of preferred heterocyclyl groups being pyridyl, quinolyl, imidazolyl and thienyl; heterocyclylalkyl, the heterocyclyl and alkyl portions of which preferably are described above; or a peptide linkage, i.e., a



group wherein k>1 and each X independently is represented by a group as described for X_1, X_2, X_3 above. Two or more linking groups can be used, such as, for example, alkyleneimino and iminoalkylene. It is contemplated that other linking groups may be suitable for use herein, such as linking groups commonly used in protein heterobifunctional and homobifunctional conjugation and crosslinking chemistry as described above. Especially preferred linking groups include amino groups which when linked to the residue of a dye via an isothiocyanate group on the dye form thiourea groups.

The linking groups can contain various substituents which do not interfere with the coupling reaction between the dye and the other components of this invention. The linking groups can also contain substituents which can otherwise interfere with such reaction, but which during the coupling reaction, are prevented from so doing with suitable protecting groups commonly known in the art and which substituents are regenerated after the coupling reaction by suitable deprotection. The linking groups can also contain substituents that are introduced after the coupling reaction. For example, the linking group can be substituted with substituents such as halogen, such as F, Cl, Br or I; an ester group; an amide group; alkyl, preferably containing from 1 to about 18, more preferably, 1 to 4 carbon atoms such as methyl, ethyl, propyl, i-propyl, butyl, and the like; substituted or unsubstituted aryl, preferably containing from 6 to about 20, more preferably 6 to 10 carbon atoms such as phenyl, naphthyl, hydroxyphenyl, iodophenyl, hydroxyiodophenyl, fluorophenyl and methoxyphenyl; substituted or unsubstituted aralkyl, preferably containing from 7 to about 12 carbon atoms, such as benzyl and phenylethyl; alkoxy, the alkyl portion of which preferably contains from 1 to 18 carbon atoms as described for alkyl above; alkoxyaralkyl, such as ethoxybenzyl; substituted or unsubstituted heterocyclyl, preferably containing from 5 to 7 nuclear carbon and heteroatoms such as S, N, P or O, examples of preferred heterocyclyl groups being pyridyl, quinolyl, imidazolyl and thienyl; a carboxyl group; a carboxyalkyl group, the alkyl portion of which preferably contains from 1 to 8 carbon atoms; or the residue of a dye.

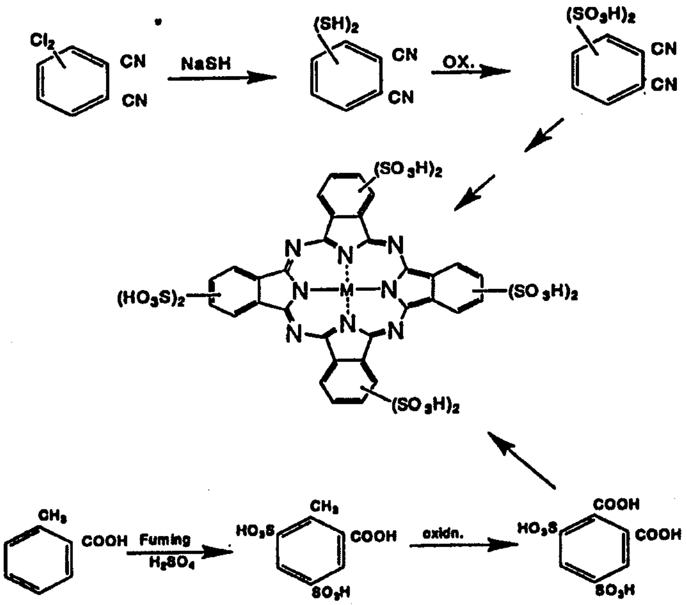
The molecular weight of the dye can be manipulated for maximum localisation at a chosen site and the presence of many localising groups in a molecule increases the likelihood that a molecule will be localised. In addition, the localisation of a single molecule of a polymeric dye built up from many individual dye molecules gives a much stronger signal than that resulting from the localisation of a single molecule of a monomeric dye. Aggregation is prevented, or substantially so, in a polymeric dye because the individual molecules in the polymer chain are constrained by the chain itself and cannot therefore align themselves in parallel to a significant extent.

The polysulphonic acid compounds used according to the invention are of particular interest in view of their ability to become immobilized in the vascular stroma of tumor tissue. This ability increases as the number of sulphonic acid groups increases. This may be due to binding to collagen, in particular uncrosslinked collagen in new tumors.

While it is possible to introduce sulphonic acid groups on preformed chromophores, it is especially preferred to introduce the sulpho groups during construction of the chromophore skeleton as the number of sulpho groups can then be better controlled. Generally this may be achieved by using chloro or polychloro substituted analogs of the aryl reagents conventionally used in chromophore construction. These can be reacted with NaSH and oxidized to the sulpho or polysulpho analogs of the conventional aryl reagents. Thus for phthalocyanines or naphthalocyanines (referred to herein by the abbreviation Pc, with PcS_n meaning such a dye with n sulphonic acid groups), PcS_8 can be constructed using disulphophthalic acids (or phthalonitriles or phthalamides, etc). Where an appropriate core forming compound is present during the synthesis, a metal or pseudometal cored $MPcS_8$ compound (where M is an element such as Si, Al, Zn, etc) can be produced.

In the case of other chromophores, it is also possible to introduce a number of sulphonic acid groups during construction of the chromophore skeleton. Thus for example with cyanines having indoles or benzindoles (or oxygen or sulphur analogs) as the end groups, cyanines with 4 or 6 sulphonic acid groups may be produced by using indole, etc reagents carrying two sulphonic acid groups. Two further sulphonic acid groups can be introduced by quaternization with propane- or butane-sulphone and additional sulphonic acid groups can be added by substitution of the central cycloalkene group, e.g., replacing a chloro substituent by a mono or polysulphonated phenol. In this way, cyanine dyes with up to 10 sulphonic acid groups per molecule can readily be prepared.

Novel monomeric dyes according to the invention which are phthalocyanines (or naphthalocyanines) with eight sulfo-groups may be prepared by the following scheme:

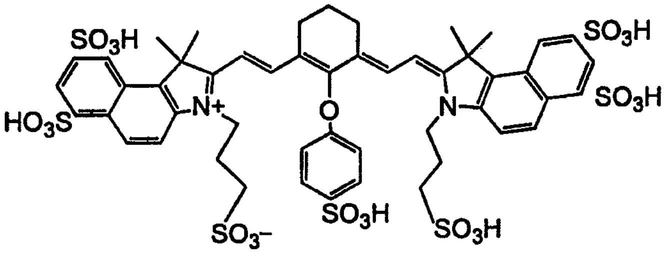


The two additional groups substituting each o-dicyanobenzene molecule may be orientated o-, m-, or p- to each other, and M represents an appropriately substituted element (e.g., Al(Cl), Si(OR)$_2$) which may optionally be present.

Compared to tetrasubstituted Pc's, some octasubstituted

Pc's have the advantage that no position isomers can be formed from two of the three possible starting materials, i.e., the 3,6 and 4,5 substituted o-dicyanobenzenes could produce only a single octasulfo-Pc derivative, increasing the yield and simplifying the purification of these products. The octasubstituted derivatives also tend to absorb at higher wavelengths, where skin penetration by light is greater. Before use these, and the other sulfonic acid derivatives discussed below, are converted to sodium or other physiologically acceptable sulfonate salts.

An example of a preferred new monomeric cyanine dye is:

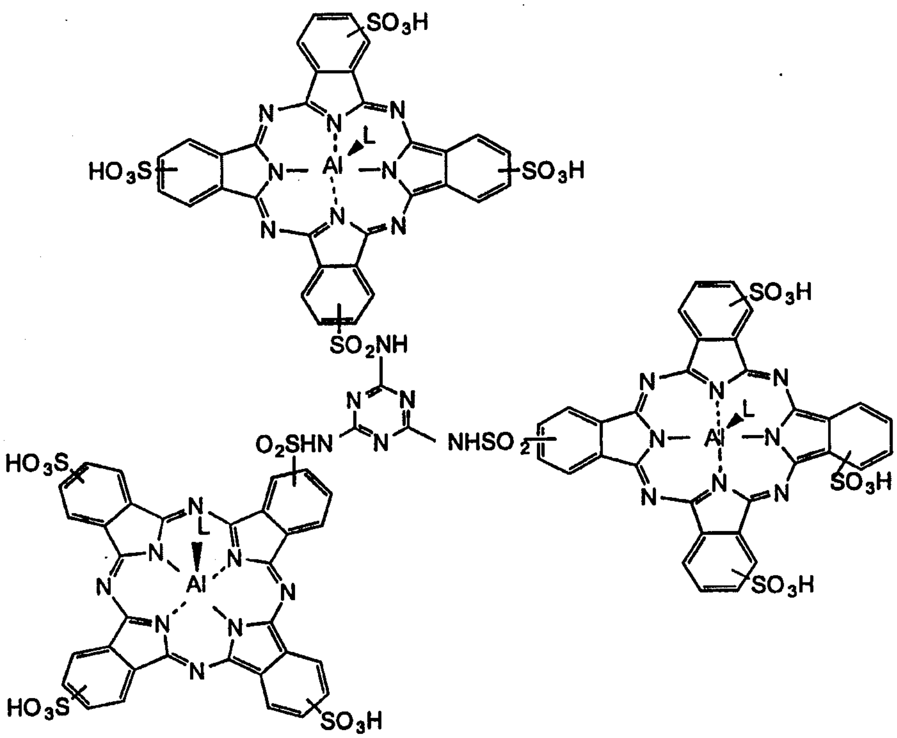


This new compound, containing seven sulfo-groups, can be made utilizing known literature methods. (Mujumdar, et al., *Cyanine-labeling reagents. Sulfobenzindocyanine succinimidyl esters*. Bioconj. Chem. 1996 7 356-62; Narayanan et al., *A new method for the synthesis of heptamethine cyanine dyes: synthesis of new near-infrared fluorescent labels*. J. Org. Chem. 1995 *60* 2391-5)

Comparable, but dye-enriched compounds can be made from di- or polyamines, e.g., from a PEG diamine:

$$_3(HO_3S)PcSO_2NHPEGNHSO_2Pc(SO_3H)_3$$

and from melamine, a triamine :



L = Cl, OH, hydrolytically stable PEG group, optionally used as linker for specific targetting vector, or other desired group.

Other polyamines that can be used include ethylenediamine, diethylenetriamine, triethylenetetramine, etc., neopentyltetramine, tris(2-aminoethyl)amine, di- and tri-aminocyclohexanes, piperazine, triazacyclononane, tetraazocyclododecane and tetradecane and pentadecane, di- and triaminobenzenes, polyaminobiphenyls, polyaminonaphthalenes, hydrazine, etc.

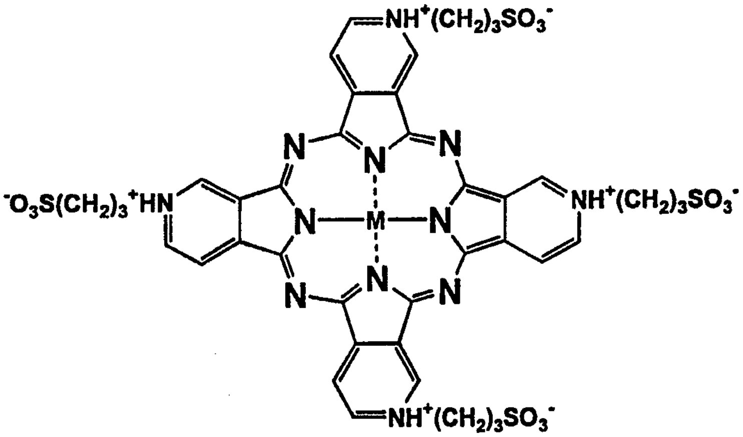
The polysulphonic acid materials discussed above are polyanionic. For some uses and for patient comfort after injection of the dyes into the blood, it may be desirable to have products that are nonionic. Such compounds can readily be prepared from acid-derived acid chlorides by reaction with amines. Where water solubility is required, hydrophilic amines are preferred, such as PEG amines, ethanolamine, diethanolamine, dihydroxypropylamines, N-methylglucamine, etc. For example, a phthalocyanine sulfonate dye PEG-based composition could be:

$$Pc(SO_2NHPEG)_4$$

where PEG = a polyethyleneglycol group of an appropriate molecular weight.

Any of the acid chloride dye derivatives described in this document which have been reacted with an amine to produce a product still possessing some unreacted acid chloride groups can subsequently be further reacted with excess of a hydrophilic amine to yield a nonionic product.

Other nonionic compositions that could be prepared include zwitterionic compounds, which would be effectively nonionic by virtue of the fact that when they are dissolved only a single solute species is produced, whereas ionic compounds produce at least two, an anion and a cation. Compounds of this type can be prepared from the well known tetrapyrido-phthalocyanines by direct tetrasulfonation but this requires forcing conditions. They may more conveniently be prepared by treatment of pyrido-Pc's with propanesultone, e.g.,



Where M = AlCl, SiCl$_2$, etc.

The positively charged nitrogen atoms can be at the 3, 4, or 5 positions in each of the isoindole groups.

This reaction with pyridine is well known (Andersen et al., Synthesis of pyridinium-1-propane-3'-sulfonate (PPS). A powerful electron scavenger and positronium inhibitor. Acta Chem. Scand. Ser. B. 1979 B33(9) 695-6) but has not previously been reported for pyridino-Pc's. The corresponding octasubstituted derivatives can be prepared from pyridazino-Pc (2 N atoms ortho), pyrimidino-Pc (2 N atoms meta), or pyrazino-Pc (2 N atoms para).

Either of these two concepts for nonionic compounds - zwitterionicity or hydrophilic amidation - could be applied to any of the dye derivatives in this disclosure to prepare nonionic hydrophilic analogs.

In addition to anionic and nonionic compounds, cationic compounds are possible, and may be of particular interest from the point of view of the good tumor localization capability of such compounds. These are also readily derived from acid chlorides, for example using a Pc tetrasulfonylchloride and cholamine one can obtain:

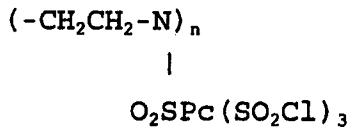
$$Pc(SO_2NH\ CH_2CH_2N^+Me_3)_4 \qquad 4Cl^-$$

The water solubility of such compounds can be improved by increasing the hydrophilicity of the quaternizing group, e.g., $-N^+(CH_2CH_2OH)_3$, $-N^+Me(CH_2CH_2OH)_2$, etc.

The above outlined types of compound can also be prepared from cyanine dyes.

The novel oligomeric and polymeric dyes according to the invention are useful compounds which can readily be synthesized, e.g., from $AlClPcS_4$, which is commercially available, and which for synthetic purposes was typically first converted to the tetrasulfonylchloride using thionyl chloride. Alternatively, the sulfonation of the Pc or other chromophore with chlorosulfonic acid and thionyl chloride gives the tetrasulfonylchloride directly as a water insoluble, readily isolable solid.

In one embodiment, products that can be oligomeric or polymeric can be made by the reaction of $Pc(SO_2Cl)_4$ derivatives with oligo- or polymeric polyamines, such as poly(ethyleneimine), etc., to form graft polymers:

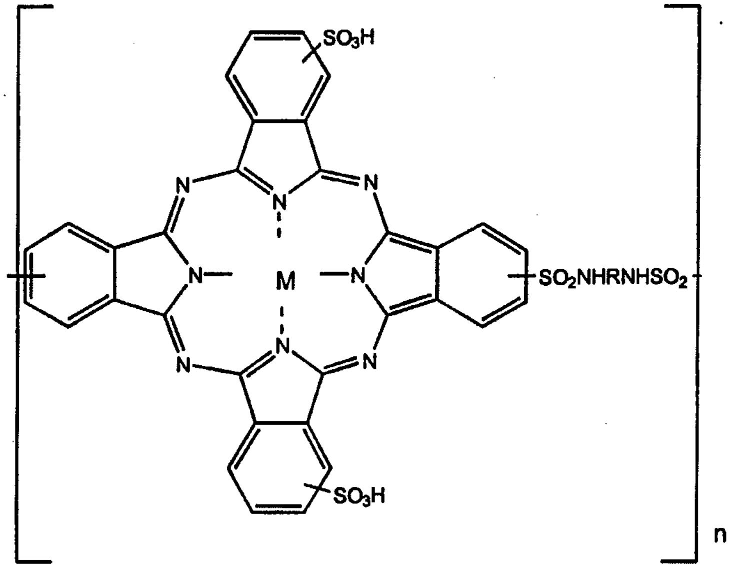


$$\begin{array}{l}(-CH_2CH_2-N)_n\\ \qquad\qquad\quad |\\ \qquad\qquad O_2SPc(SO_2Cl)_3\end{array}$$

If desired, other reactions can be carried out at this stage or, if an anionic product is desired, a final hydrolysis step generates the required polysulfonamide. It is recognized that the reaction product depicted is oversimplified and that pendant $-NH_2$ groups may be present in the starting polymer, and that branching and crosslinking may occur, but this does not affect the efficacy of the products as long as many unreacted sulfo-groups are present in the final product.

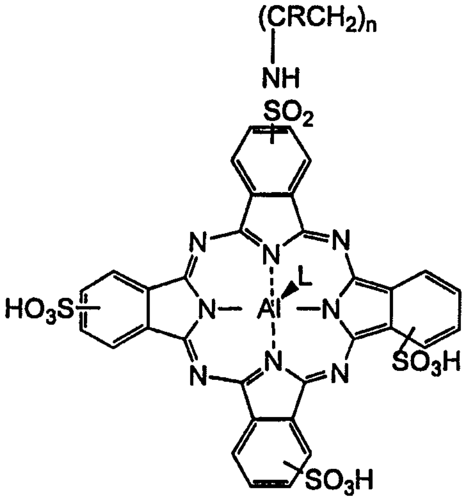
This can be achieved by control of the relative proportions of the two reactants.

Dyes with sulfonamide Pc groups in the backbone can be made as oligomers or polymers by reacting PcS_4 sulfonyl chlorides with diamines in equimolar proportions (copolymerization):



where R represents ethylene to poly(ethylene), di(ethyleneoxy) to poly(ethyleneoxy), phenylene to polyphenylene, etc.

Polymeric or oligomeric dyes with pendant sulfo-Pc groups can be prepared by vinyl polymerization of a Pc containing monomer, e.g., from an acrylic sulfonamide:



where R = H or lower alkyl

A similar product can be made by reacting the Pc tetrasulfonylchloride with poly(allylamine) or with poly(vinylamine).

The invention also covers the use of carboxamide derivatives analogous to those described above, which can be prepared for example from Pc tetracarboxylic acid chlorides. Related materials can also be prepared from tetrahydroxy-Pc's and polyacid chlorides (e.g., from acrylic acid) or polyanhydrides (e.g., from maleic anhydride). Comparable products may also be prepared from cyanine and other dyes.

Particularly interesting dyes usable according to the invention include cofacial phthalocyanine derivatives, including oligomers and polymers. These are materials that are linked through the element in the center of the Pc molecule, and this element accordingly needs to be tri- (e.g., Al, Ga) or tetravalent (e.g., Si, Ge, Sn) in order to have axial groups available for linkage. Only the tetravalent elements would have the two axial groups needed for polymerization. Any groups could be present at the periphery of the Pc system (e.g., $-SO_3Na$) provided that they would not interfere with the polymerization chemistry, hence products of this type could be either ionic or nonionic (or have the potential to be made so). Hence, for the Si compound:

$$PcSi(OH)_2 + ClSiMe_2(CH_2)_3NCO \text{ ------------> } PcSi[OSiMe_2(CH_2)_3NCO]_2$$

This diisocyanate forms polyurethane oligomers and polymers with diols e.g., ethylene glycol and other $HO(CH_2)_nOH$, or with $HO(CH_2CH_2O)_nOH$ (PEG), and it could also be reacted with analogous diamines to form polyureas.

The corresponding isothiocyanate can also be prepared and similar chemistry performed.

AlPc compounds could not be used for cofacial polymeric products having more than two chromophores, but two such molecules could be cofacially linked, using chemistry analogous to that described above, to produce the dimeric product which is a polymer for the purposes of this text.

The dye compounds used according to the invention may be administered by any convenient route, for example by injection or infusion into muscle, tumor tissue, or the vasculature, subcutaneously, or interstitially, by administration into an externally voiding body cavity (e.g., into the digestive tract (for example orally or rectally), vagina, uterus, bladder, ears, nose or lungs), by transdermal administration (e.g., by iontophoresis or by topical application), or by topical application to a surgically exposed site.

In general, parenteral administration, e.g., of a solution or dispersion of or containing the dye compound, will be preferred.

The administration forms used may be any of the forms conventionally used for administration of pharmaceuticals, e.g., solutions, suspensions, dispersions, syrups, powders, tablets, capsules, sprays, creams, gels, etc.

The dye compound, if water-soluble, can be administered in the form of an aqueous solution. Alternatively, and in many cases preferably, the dye compound may be presented in particulate form, e.g., liquid droplets of or containing the dye (e.g., in solution in a water-immiscible fluid), or solid or semi-solid particles of, containing or coated with the dye. This latter category includes vesicles (e.g., liposomes, micelles or microballoons) containing the dye compound.

Because of their intrinsic light scattering effect, particles are a preferred presentation form for the dye. Particle sizes may range from a few nanometers to about 20 micrometers, e.g., 10 to 20000 nm. However the larger particle sizes (above 10 micrometers) will generally only be used with deformable particles.

Where the particles contain other components besides the dye compound, e.g., matrix or membrane forming materials, coating agents, contrast agents, solvents, gases or gas generators, etc, these will conveniently be materials which are physiologically tolerable at the dosages used. The formation of droplets, coated particles, composite particles, vesicles, etc is well described in the literature, especially that relating to pharmaceutical and contrast agent (e.g., ultrasound contrast agent) preparation and formulation.

Where a water-soluble dye is to be used to mark tumors for surgical removal, then it may be preferred to administer the dye in particle form in order to reduce staining of, and hence removal of, healthy tissue surrounding the tumor. Indeed in place of conventional water soluble dyes such as methylene blue, it may be desirable to use derivatised analogs, where derivatisation has been to introduce lipophilic groups which will reduce leakage of the dye from the particle, e.g., an emulsion droplet or a liposome. For this purpose, one may use long chain hydrophobic groups, such as C_{10-30} alkyl or alkenyl chains, preferably groups of a similar length to the lipophilic component of any liposome membrane forming material that is present in the particle. This type of chromophore derivatization can be made for any dye.

It may be preferred to inject the dyes of the present invention directly into the tumor. Thus it is possible to directly inject dyes of the present invention or suspensions of insoluble dyes of the present invention in order to stain tumors before surgical resection. The dyes will mark the tumor to the margins for an extended period of time such that surgical resection is facilitated or for PDT.

Such particulate suspensions of the dye afford an advantage over conventional dye solutions in terms of residence times within the tumor. A further advantage over conventional intravenous administration of the dyes is that the direct injection technique removes the need for a vector to deliver the dye to the tumor site. Furthermore, the need to use physiologically safe dyes is reduced since systemic exposure is obviously limited via the direct injection rather than the intravenous route.

Dyes used in the direct injection technique are preferably those which absorb light and/or fluoresce in the visible region. However, dyes which fluoresce in the near-IR region could also be useful in this technique due to the enhanced tissue penetration of such emitted light. In the latter case, surgeons could utilise IR-sensitive imaging equipment (i.e., "night vision" goggles) during surgery to locate the fluorescing dyes within the tumor tissue.

Furthermore, dyes used in the direct injection technique can additionally be iodinated. In this manner, post-surgical radiation therapy would be enhanced due to the enhanced radiation uptake in the remaining tumor tissue due to the presence of the iodinated dye.

Thus while many of the dye compounds described herein are water soluble and can therefore be administered by injection, they can also be incorporated into liposomes and injected in this form. Dyes that are very water soluble can be chemically modified prior to liposome incorporation.

Alternatively, these dye compounds may be administered via the oral route for absorption through the lining of the stomach, the intestines, and the colon, see for example, Carrier-mediated intestinal transport of drugs, Tsuji, A.; Tamai, I., Pharmaceutical Research (New York) Vol. 13, No. 7, p. 963-977, 1996; Oral protein drug delivery, Wang, Wei, J. Drug Targeting Vol. 4, No. 4, 1996, pp. 195-232; Improved passive oral drug delivery via prodrugs, Taylor, Michael D., Adv. Drug Delivery Rev. Vol. 19, No. 2, 1996, pp. 131-148; Oral colon-specific drug delivery: a review, Van den Mooter, Guy; Kinget, Renaat, Drug Delivery, Vol. 2, No. 2, 1995, pp. 81-93; Present status of controlled drug delivery system-overview, Naik, S.R.; Shanbhag, V., Indian Drugs, Vol. 30, No. , Sep 1993, pp. 423-429; Novel formulation strategies for improving "oral" bioavailability of drugs with poor membrane permeation or presystemic metabolism, Aungst, B.J., Journal of Pharmaceutical Sciences (USA), Vol. 82, No. Oct 1993, pp. 979-987; Remington's Pharmaceutical Sciences, A. Osol, ed., Mack Publishing Co. 1975, Part 6, chp 40 and references therein. (pp 731-753), Part 8, all chps (pp 1355-1644); The Extra Pharmacopoeia, Martindale, 29th Edition, The Pharmaceutical Press, London, 1989.

Administration of drugs and other agents by this route is often preferred due to enhanced patient compliance (for repeated dosing) and ease of administration. It is well known in the art that not every agent is bioavailable via this route; that is to say, that not all molecules are 1) chemically stable in the environs of the gut, 2) transportable across alimentary membranes for absorption into the blood/lymphatics, and 3) active even if accessible due to metabolic processes within the gut or possible solubility issues, etc. However, it is also known in the art, that alteration of the molecular structure to control the relative hydrophobicity of the molecule (such as, partition coefficient between octanol and water or log(P)) within a preferred range can increase the oral availability of the agent.

Additional methods of administration include the following: air guns, inhalation, aerosols, body cavity washing (i.e., enemas), and tissue marking devices (i.e., pens and tattoo needles).

It is also known that the addition of penetration enhancers such as some fatty acids, some bile salts, and other lipophilic compounds can enhance the bioavailability of the drug/dye of interest. Further, the use of cyclodextrins may alter the penetration and bioavailability of normally non-available dyes by the oral route. Where the dye of interest incorporates a metal ion, it has been shown that the addition of adjuvants such as salicylic acid and citric acid can significantly enhance the oral availability of the metal complex . Formulating the dye of interest in a polymeric composition which effectively "coats" the lining of the gut will also enhance the availability of the dye by artificially increasing the concentration of the dye at the lining of the gut.

These agents can also be administered via body cavities such as the mouth, the rectum, the vagina, the peritoneal cavity (i.e., by intraperitoneal injection or cavity washings such as enemas), etc. as well as topical, intramuscular, subcutaneous, and pulmonary administration. All of the known routes of administration of drugs/agents to mammals are envisaged according to the present invention.

While the dye compounds of the invention are particularly suited to intraoperative and post-surgical PDT use to facilitate visualization of tumor margins and optimize the surgical removal and PDT destruction of tumor tissues and cells, they can also be used as contrast agents in light imaging of tumor tissue, tumor cells and diseased lymph nodes. In addition, the dye compounds of the invention are suited to the following medical uses: removal of arterial plaque, burn treatment, cauterization, cryosurgery/hyperthermia (dye guidance of the administration of cold/heat), treatment of dental/gum diseases, dermabrasion (use of a wire brush, for example, to remove cells), directing the application of chemotherapeutic agents, measurement of dynamic flow or tissue perfusion, electrolysis, electrophoresis, application of embolic agents, detection and treatment of endometriosis, tissue implantation (dyes contained in implants can be used to monitor the integrity of the implant), fluid marker (dyes can be added to bodily fluids to monitor the flow, location, swelling etc.), focused ultrasound therapy (dyes guide the placement of the ultrasound transducer), marking sites of inflammation and infection, lavage therapy, lymph node detection, microwave therapy, MRI in real time (this having neurosurgical applications), marking nerve bundles, marking normal tissue and thus highlighting non-normal tissue, such as tumorous tissue, marking neural tumors, oncology procedures (such as tumor marking and lymph node involvement), detection of pathological conditions (i.e., tumors, lumps, cysts (both benign and cancerous)), peripheral metastases localization, photodynamic therapy, plastic and cosmetic surgery, radiation therapy (dyes can guide the placement of radiation equipment), sonodynamic therapy, surgical plane marking (dyes mark boundaries between tissue groups to allow surgeons to between instead of through a tissue group), tissue marking and tissue marking pens (dyes can be used in devices which draw or mark sections of tissue), tumor marking (dyes mark tumor sites using either passive or active targeting), vascular leaks (dyes can be used to determine the vascular integrity after the repair of vascular damage such as haematomas), vasectomies and tubal ligations (the dyes can be used to determine the integrity of any repairs) and for white blood cell markers.

One way to use dyes intraoperatively is in the visualization of dynamic flow or tissue perfusion. In this method, the tissue (generally a tumor) is exposed and the dye is then administered intravenously. Depending on dye pharmacokinetics, the agent will generally begin in the blood pool, leak into the tumor, and then gradually fade as the dye is excreted or leaks into the surrounding tissue. Dynamic agents could help doctors discriminate between the necrotic core and actively growing tumor regions.

Dyes attached to capillary embolic agents can be used for light imaging purposes. Capillary embolic agents are a novel class of embolic agents which embolize the entire capillary bed of tumors or tissue malformations rather than simply blocking the feeding artery to those tissues or tumors. The dye or fluorescent agent can then be treated as a separate "drug" and delivered to the tumor by the X-ray, MRI, or ultrasound dense particles and trapped at the tumor site after embolus formation. In this manner, the tumor or lesion can be "marked" for ease of resection without the removal of normal tissue.

While both normal and pathological tissue can be marked to aid in removal, in reconstructive surgery tissue is not necessarily removed. Rather, boundaries are marked in order to aid in the repair. Plastic surgery is therefore another use for dyes of this invention and is defined herein as the restoration, reconstruction, correction, or improvement in the shape and appearance of body structures that are defective, damaged or misshapen by injury, disease, growth and development, aging, previous surgeries and environmental stresses. By way of example, tissue repair would include treating burns victims, repairing congenital defects and reversing sterilization procedures such as vasectomies and tubal ligations. Plastic surgery also includes the subdivision of cosmetic surgery, which is defined herein as procedures designed to improve the patient's appearance by plastic restoration, correction, removal of blemishes etc.

A further use for dyes of this invention is in regard to surgical planes. Surgeons would like to have dyes which mark the margins of surgical planes. When operating, surgeons try to pass between tissue groups rather than through them but in some cases it is not always easy to see where the edge of one tissue group ends and another begins. Thus a dye that would enhance this demarcation would be particularly useful. By way of example, a dye whose distribution is controllable could be injected locally (i.e., subcutaneously or iontophoretically) in the area that the surgeon requires demarcated. Diffusion could be controlled iontophoretically so that when it came time for surgery, the dye would be diffused along the surgical plane, thus allowing the surgeon to visualize the appropriate areas. Alternatively, dyes with different hues (such as dyes containing different metals) could be individually sprayed or injected onto a surgical plane. Once the surgeon reached non-dyed tissue (such as tissue in another plane) a dye with a different hue would be spritzed or injected into the new area.

There are also times when the surgeon will have to remove normal tissue. Both pathological and healthy normal tissue can be marked with a dye in order to remove tissue. One example is liposuction. The fatty tissue to be removed in such a procedure does not have to have pathological conditions associated with it. A problem with the liposuction procedure is that often non-fatty tissue is also removed. A dye of the present invention could be used to stain either the fat or the non-fatty tissue offering visual clues to the surgeon as to when the suction step was leaving the fatty tissue behind. A further example is the removal of hair using electrophoretic techniques.

Furthermore, in addition to the above, it is often desired to remove tissue that is neither cancerous nor "normal". By way of example, a pituitary gland might be over-producing hormones which interfere with the normal development of the body and hence need to be removed. Dyes of the present invention would help differentiate such non-"normal", non-cancerous tissue groups.

A further use for the dyes of the present invention concerns their use in conjunction with another imaging or therapeutic agent. Such combinations will have the advantage of reducing the imaging and site-marking process to a single step, the dye being incorporated into the "standard", i.e., stand-alone, imaging or therapeutic agent. Therefore the dye need not be injected after the imaging or therapeutic agent is injected. One possible use for such imaging agent/dye combinations is in the determination of the spread of melanoma cancer. Traditionally, in order to determine the spread of such, a radioactive probe is injected into the lymph system associated with the melanoma and this is followed by an injection of dyes into the lymph nodes. Using imaging agent/dye combinations as described above, the dye and the radioactive probe will be combined together, thus reducing the procedure to a one-step process.

Furthermore, it is possible to use dyes of the present invention in contrast agents capable of generating multiple reporting signals (the signal being reported can be radiation, relaxivity (in MRI, for example), ultrasound or light (where a change in refractive index, phase, frequency, amplitude, intensity, polarisation, and the like can be monitored)).

Many existing patents disclose the use of a given chelator agent which can be used in, e.g., MRI (with Gd) or light (with Eu) studies). However dyes of the present invention can be used synergistically in multiple modalities. By way of example, agent can be used in one mode, e.g., as an MRI agent before surgery (i.e., in a non-invasive mode) to confirm the existence of a diseased state/structure. Then, during the actual surgical intervention, another mode (built into the contrast agent) would be used to guide the surgery, such as localize the tissue visually, demarcate tissue boundaries, differential staining of tissue (e.g., surgical planes) and/or use of different wavelengths to highlight functional biological aspects. Such contrast agents can be coupled to vectors such as proteins, peptides, DNA, polymers and the like.

The light imaging technique may involve recording a photographic image of a tissue or organ surface illuminated with a light source that causes the contrast agent to exert a contrast enhancing effect and make the unhealthy tissue stand out more clearly from the surrounding healthy tissue, e.g., due to characteristic light absorption or fluorescence by the contrast agent. Such a photographic image may be recorded of an exposed surface (e.g., exposed during surgery) or may be recorded by insertion of an endoscope into a body cavity through a body orifice or through a surgical incision.

Alternatively, images may be recorded of light (generally laser light, especially at near infrared wavelengths) reflecting from or transmitting through body tissue. In these techniques, the use of a contrast agent facilitates detection and location of subsurface structures, e.g., tumors.

Other light imaging techniques may be used to record contrast enhanced images of shallow subsurface structures, e.g., within a millimeter of the surface.

Confocal scanning laser microscopy (CSLM) selectively images a single point within a test object by focusing light from a pinhole source onto that point. The light transmitting past or reflecting from that point is then refocused onto a second pinhole that filters out spurious light. Thus the detector behind the pinhole picks up the image of the point alone. Raster scanning of the point through a complete plane passing through the sample with the help of movable mirrors generates the image of a complete plane of points. Thus, CSLM is a means for "optically" sectioning a test sample.

Optical coherence tomography (OCT) accomplishes optical sectioning in a related, but somewhat different manner. A collimated beam of light is reflected from the sample, then is compared with a reference beam that has traveled a precisely known distance. Only the light that has traveled exactly the same distance to the sample and back as has the reference beam from the source to the detector constructively interferes with the reference beam and is detected. Thus, the light from a single plane within the sample is again selected.

CSLM and OCT are particularly suitable for examination of excised tissue. However, they could also be used for in vivo optical biopsy of suspicious skin lesions and for characterization of body surfaces that can be exposed endoscopically or surgically.

It is anticipated that CSLM and OCT will be especially useful in optically guided tumor resection. For example, either device attached to a colonoscope will facilitate determination of the appropriate depth to which a malignant colon polyp should be excised to ensure complete removal of cancerous tissue. Additional applications include, but are not limited to, diagnosis and treatment of disease conditions of the rest of the digestive tract, surgical treatment of ulcerative colitis, and diagnosis and treatment of endometriosis.

When either CSLM or OCT is used as a dermatological tool, scattering from melanosomes in the skin is believed to be responsible for producing contrast. The primary effect of these sites is to scatter or reflect light hitting them. Synthetic contrast agents can act either as scattering or absorbing agents.

A preferred contrast agent for intraoperative CSLM or OCT will have the following properties: It will consist of stabilized particles in aqueous or buffered solution. The particle size will be around 200 to 1300 nm (roughly equal to the wavelength of the light source). The refractive index of the particles will differ from that of body fluids such as blood and lymph by at least 0.01. The particles may be made of a dye compound or may contain or be coated with a dye compound, e.g., the particles may comprise a matrix material (e.g., a physiologically tolerable synthetic or non-synthetic polymer, such as an acrylate or polysaccharide) incorporating a dye compound, a core of a dye compound coated with a coating agent or encapsulated by a membrane forming material, or a core of a matrix material with a dye compound coated on or attached to the particle surface. The particles may be solid, semi-solid or liquid and may be layered structures such as vesicles (e.g., micelles, liposomes and microballoons). The dye compound used will preferably be a fluorescent material, particularly a material having an emission maximum in the near infrared range, especially in the range 650 to 1300nm. Optionally the particles may have suitable surface modifying agents, such as poly(ethylene glycol) to slow their uptake by macrophages in the body. Examples of suitable particulate agents are described in WO 96/23524.

The dye compounds to be used as contrast agents in methods of light imaging according to the invention, e.g., in conjunction with CSLM or OCT, may also be polymeric or monomeric water-soluble dyes, e.g., as described herein.

Additional imaging methods which can be used with the dye compounds of the present invention include two-photon and multiphoton fluorescence microscopy, endoscopy (bronchoscopy, colonoscopy, laparoscopy), fluorescence spectroscopy, frameless stereotaxy, intraluminial photothermoforming (see US-A-5662712), intraoperative angiography, intraoperative Doppler, methods utilising polarized light or UV lamps, methods utilizing vision augmentation devices (e.g., night vision goggles, cameras, filters, computer imaging enhancement, virtual imaging devices, magnifying devices, prisms and polarizers) and scintigraphy.

The contrast agent can be injected into the vasculature prior to or during surgery. For detection of lymph nodes it can be injected into a lymph duct draining into the surgical area. Alternatively, it may be applied during surgery as a topical ointment, a liquid, or a spray.

For dermatological applications, the contrast agents described in WO 96/23524 may be modified to be delivered through transdermal patches or by iontophoresis. Iontophoretic delivery is preferred, as one can control the amount of the agent that is delivered.

In some situations the resolution of *in vivo* techniques such as CSLM and OCT may be high enough to allow the separation of the images of the nuclei and cytoplasm of individual cells. A contrast agent that specifically localizes in the nucleus or the cytoplasm of cells will increase the contrast between these sites and that of the background. Agents for this purpose will include non-toxic forms of staining dyes already used for histological examination of excised tissue. A list of such selectively staining dyes can be found in any handbook of histological stains.

The perfusion of tissue that is exposed by surgery is one important indicator of the health of that tissue. One of the indicators of the degree of perfusion is the rate of blood flow within the tissue. Blood flow in the skin can be detected by laser Doppler blood-flow measurement or laser speckle interferometry, either by itself or in conjunction with CSLM or OCT.

Laser Doppler and speckle interferometry are related, and each relies upon the fact that the intensity of light detected after a beam of laser light that interacts with a collection of moving particles changes with time (Ruth, B., "Blood Flow Determination by the Laser Speckle Method," *Int. J. Microcirc.: Clin. Exp.*, **1990**, *9*, 21-45). Mathematical analysis of the changes provides a basis for calculating the rate at which the particles are moving.

When laser light with a wavelength between 300 and 1300 nm is reflected from the skin, the changing pattern of light reflecting back to a light detector results largely from movement of blood cells within the dermis. However, speckle interferometry is best suited for determination of blood flow in vessels with diameters between 0.08 and 1 mm. Laser Doppler measurement is best used for blood vessels 0.08 mm or less in diameter (Ul'Yanov, S.S.; Tuchin; Bednow; Brill, G.E.; Zakharova, E.I., "The Application of Speckle Interferometry for the Monitoring of Blood and Lymph Flow in Microvessels", *Lasers in Medical Science*, **1996**, *11*, 97-107). Light reflected from larger vessels, which lie deep within the dermis and in the underlying tissue, only serves to complicate the analysis of the light from the smaller vessels lying about 0.5 mm beneath the surface of the skin (Abbot, N. C.; Ferrell, W. R.; Lockhart, J. C.; Lowe, J. G., "Laser Doppler Perfusion Imaging of Skin Blood Flow Using Red and Near-Infrared Sources", *J. Invest. Dermatol.*, **1996**, *107*, 882-886).

When the vasculature contains an absorbing dye, Beer's law shows that light passing through larger blood vessels will be more strongly attenuated than is light passing through smaller vessels. Furthermore, light scattering from blood cells in vessels deep within the skin and underlying tissue will be more attenuated than will light scattering from blood cells in vessels near the surface of the skin. Thus, the presence in the blood of a contrast agent with an absorption maximum near the wavelength of the laser light used for either Doppler or speckle interferometric measurements of blood flow improves the selectivity of the measurement for smaller vessels near the skin surface. An agent of the type taught by this invention is necessary as there must be a stable concentration of the agent in the blood over the course of the measurement.

The dyes of the invention may be administered to patients for imaging in amounts sufficient to be visualizable or to be effective in PDT in the particular surgical technique.

The dosage of the dye compounds of the invention will depend upon the condition being treated, but in general will be of the order of from 1 pmol/kg to 1 mmol/kg bodyweight.

The dye compounds of the present invention may be formulated with conventional pharmaceutical or veterinary aids, for example emulsifiers, fatty acid esters, gelling agents, stabilizers, antioxidants, osmolality adjusting agents, buffers, pH adjusting agents, etc., and may be in a form suitable for parenteral or enteral administration, for example injection or infusion or administration directly into a body cavity having an external escape duct, for example the gastrointestinal tract, the bladder or the uterus. Thus, the compounds of the present invention may be in conventional pharmaceutical administration forms such as tablets, capsules, powders, solutions, suspensions, dispersions, syrups, suppositories etc. However, solutions, suspensions and dispersions in physiologically acceptable carrier media, for example water for injections, will generally be preferred.

The dye compounds according to the invention may therefore be formulated for administration using physiologically acceptable carriers or excipients in a manner fully within the skill of the art. For example, the compounds, optionally with the addition of pharmaceutically acceptable excipients, may be suspended or dissolved in an aqueous medium, with the resulting solution or suspension then being sterilized.

For some portions of the body, the most preferred mode for administering the dye compounds is parenteral, e.g., intravenous administration. Parenterally administrable forms, e.g., intravenous solutions or dispersions, should be sterile and free from physiologically unacceptable agents, and should have low osmolality to minimize irritation or other adverse effects upon administration, and thus the contrast medium should preferably be isotonic or slightly hypertonic. Suitable vehicles include aqueous vehicles customarily used for administering parenteral solutions or dispersions such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride

Injection, Lactated Ringer's Injection and other solutions such as are described in Remington's Pharmaceutical Sciences, 15th ed., Easton: Mack Publishing Co., pp. 1405-1412 and 1461-1487 (1975) and The National Formulary XIV, 14th ed. Washington: American Pharmaceutical Association (1975). The solutions or dispersions can contain preservatives, antimicrobial agents, buffers and antioxidants conventionally used for parenteral solutions, excipients and other additives which are compatible with the dyes and which will not interfere with manufacture, storage or use.

All of the publications referred to herein are incorporated herein by reference.

Any mention in the text to the term "mean MW" or "mean molecular weight" refers to the weight average.

The invention will now be described further with reference to the following non-limiting Examples and Figures. In the Examples, the abbreviation Pc is used to refer to phthalocyanine, optionally metallated.

Figure 1 is a graph of comparative biodistribution data of contrast agent Polymer 1 versus chloroaluminum phthalocyanine tetrasulfonate as control in female immunodeficient mice containing HT-29 tumors at one hour post intravenous injection of phosphate buffered saline solutions of each. Polymer 1 is detected in the tumor; the control compound is negligibly detected.

Figure 2 is a graph of comparative biodistribution data of contrast agent Polymer 2 versus chloroaluminum phthalocyanine tetrasulfonate control in female immunodeficient mice containing HT-29 tumors at one hour post intravenous injection of phosphate buffered saline solutions of each. Polymer 2 is detected in the tumor; the control compound is negligibly detected.

Figures 3A is a graph of comparative biodistribution data of contrast agent Polymer 3 (NC100448) versus indocyanine green as control in female immunodeficient mice containing HT-29 tumors at one hour post intravenous injection of phosphate buffered saline solutions of each. Polymer 3 is detected in the tumor; the control compound is negligibly detected.

Figures 3B is a graph of comparative biodistribution data of contrast agent Polymer 3 (NC100448) versus indocyanine green as control in female immunodeficient mice containing HT-29 tumors at three hours post intravenous injection of phosphate buffered saline solutions of each. Polymer 3 is detected in the tumor; the control compound is negligibly detected. Relative to Figure 3A, the concentration of the contrast agent in the tumor has increased while the concentration in the blood has decreased.

Figure 4 is a photograph taken under room fluorescent light of female immunodeficient mice with exposed human HT-29 tumors 24 hours post intravenous (tail vein) injection with 0.1 mg/(g body weight) in PBS of the blue contrast agent NC100526. The labeled sheet to the right of the mice was spotted with PBS solutions of several contrast agents of this invention and then dried in order to show how they appear under various illumination and imaging conditions. The tumors (indicated by arrows) have been sliced to expose their interiors as well as their exteriors. The tumors exhibit a blue color due to the presence of contrast agent, and have areas of darker color due to increased concentrations of contrast agent present along the tumor edges (as indicated by arrows in the black and white photograph) and in some regions of the interior.

Figure 5 is a photograph which shows a comparison of tumors surgically removed from mice treated as in Figure 4 with contrast agent NC100526 versus tissues to which the tumors had been attached. The arrow at the left points to a region of contrast agent detectable within the tumor by its blue color; the arrow at right points to a region of contrast agent detectable by its blue color on the surface of the tumor. The residual tissue had a normal color.

Figure 6 shows that the contrast created by the presence of contrast agent NC100526 at the outside edge of the tumors pictured in Figures 4 and 5 (arrows) is enhanced when the image is recorded with Kodak high-speed black and white infrared film. The light source was ambient fluorescent room light.

Figure 7 shows a comparison image of tumors taken from mice 24 hours after they were injected with 0.05 mg/(g body weight) of the infrared contrast agent NC100448 in PBS. The photograph was taken with black-and-white infrared film using irradiation at 780 nm from an array of light-emitting diodes. These conditions gave maximum absorption and contrast enhancement of NC100448 as evidenced by the darkened spot of contrast agent on the paper chart. The tumors appear uniformly darker than control tumors (not shown), but do not show darkening around the edges as seen in tumors from mice injected with NC100526 (Figure 4). Measured biodistribution of the contrast agent in the mice (see Table 1) shows that the tumors contain NC100448.

Table 1 presents biodistribution data at 3, 6, and 24 hours, reported as per cent injected dose of the contrast agent per gram of tissue, from a separate experiment of mice intravenously injected with contrast agent NC100448 at 0.05 mg/(g body weight) in PBS. At 24 hours the concentration of contrast agent in the tumors remained relatively high while the contrast agent had largely cleared from the blood and had not increased in the muscle.

Table 1

NC100448 Biodistribution Data in HT 29 Tumored Mice

	Plasma	Tumor	Muscle
3 hr	14.33±1.33	5.57±0.73	nd
6 hr	8.44±1.33	8.6±1.53	nd
24 hr	0.46±0.40	7.53±0.74	nd

nd = none detected

Figures 8a and 8b are photographs of two views taken from different angles of HT-29 tumors sliced open and removed from mice at 24 hours post injection with 10 mg/(g body weight) of NC100505 in PBS and irradiated with 366 nm wavelength light. The tumors, two of which are indicated by arrows in Figure 8a, are brightly fluorescent with a yellow color. In the black and white photograph they appear as bright spots. Other organs and the skin appear normal.

Figures 9a and 9b show two photographs of a control mouse with two excised tumors. Under ambient fluorescent room light or 366 nm light the tumors have a color similar to that of the skin.

Figure 10a is a photograph of a collection of excised tumors viewed under ambient fluorescent room light. The top row contains tumors resected from mice at 24 hours post i.v. injection with NC100505 in PBS. The second, third, and fourth rows are tumors resected from mice 24 hours post i.v. injection with PBS solutions of NC100526, NC100448, and NC100524, respectively. At the center to the right is a tumor from the control animal. Under ambient fluorescent room lights the tumors in the second row were clearly blue while the other tumors appeared similar to the control tissue. The shading difference is barely perceptible in the black and white picture shown but is very distinct in the original color photograph.

Figure 10b is a photograph of the same collection of excised tumors as seen in Figure 10a except that they were irradiated at 680 nm and photographed with Kodak Ektachrome EIR high speed color infrared film. (The label reading 366 nm is in error and has been struck out with an arrow.) Under this incipient light close to the absorption maximum of NC100526, the tumors in the second row from the top clearly appear darker than the other tumors.

Figure 10c is a photograph of the same collection of excised tumors as that pictured in Figure 7 but photographed under irradiation with 366 nm light. The tumors in the top row fluoresced brightly and appeared yellow while the other tumors did not fluoresce under these conditions.

Figure 11 is a photograph of a set of excised tumors irradiated with 780 nm infrared light that were removed from mice 24 hours post injection with PBS solutions of NC100448 (top row) and indocyanine green (bottom row). The image was recorded with an infrared sensitive CCD camera (Bischke, Type N-CCD-4024, fitted with a Computar TV Zoom lens 1:1.2/12.5-75) on VHS tape (Maxell HGX Plus) at 30 frames/sec using a Panasonic AG-1310P HQ Video Cassette Recorder with super 4-Head VHS SQPB). The tape was digitized and one frame was printed using Adobe Photoshop 4.01. Tumors from mice injected with NC100448 (absorption maximum approximately 780 nm) appeared significantly darker when compared to tumors from mice injected with ICG.

Table 2 shows the biodistribution, reported as per cent injected dose of the contrast agent per gram of tissue, at several time points after injection of NC100481 in female immunodeficient mice with HT-29 tumors. Whereas within 24 hours the contrast agent has cleared from both the blood and muscle, it remained at a high concentration in the tumor after 48 hours.

Table 2

NC100481 Biodistributions

	Plasma	Tumor	Muscle
6 hr	7.4±0.3	21.0±7.1	10.3±17.9
24 hr	nd	16.6±3.9	nd
48 hr	nd	9.1±4.5	59.5±42.9

nd = none detected

Figure 12. Pre-Injection MRI Lymphography

NC 100503; an introperative light imaging contrast agent comprised of both fluorescent dye moieties and gadolinium chelators with molecular weight > 20000 was administered subcutaneously into the dorsal side of the rear paws of rabbits. This image represents the region of the popliteal lymph nodes before administration of the contrast agent. The images were taken as spin echo images with TR = 300 ms and TE = 15 ms using a GE Sigma 1.5 Tesla imager at the Stanford Veterans Administration Hospital, Palo Alto, CA. The field of view was 12 x 12 cm using a grid of 256/128 pixels per image. The linear white areas rising in each leg are fatty deposits leading up to the lymph nodes themselves which are not visible on the right side of this image but can be "seen" as dark spaces within the fatty streaks on the left side.

Figure 13. MRI Lymphography 10 Minutes Post Injection of NC 100503

NC 100503; an introperative light imaging contrast agent comprised of both fluorescent dye moieties and gadolinium chelators with molecular weight > 20000 was administered (1 ml) subcutaneously into the dorsal side of the rear paws of rabbits. This image represents the region of the popliteal lymph nodes 10 minutes after administration of the contrast agent. The images were taken as spin echo images with TR = 300 ms and TE = 15 ms using a GE Signa 1.5Tesla imager at the Stanford Veterans Administration Hospital, Palo Alto, CA. The field of view was 12 x 12 cm using a grid of 256/128 pixels per image. The spaces which were dark or not visible in the pre-injection image are now extremely white signaling the presence of the gadolinium chelator portion of NC 100503 present within the nodes. Especially noticable is the transformation on the left side of image from dark spaces to intense white due to the contrast agent.

Figure 14. MRI Lymphography 2 Hours Post Injection of NC 100503

NC 100503; an introperative light imaging contrast agent comprised of both fluorescent dye moieties and gadolinium chelators with molecular weight > 20000 was administered (1 ml) subcutaneously into the dorsal side of the rear paws of rabbits. This image represents the region of the popliteal lymph nodes 2 hours after administration of the contrast agent. The images were taken as spin echo images with TR = 300 ms and TE = 15 ms using a GE Signa 1.5Tesla imager at the Stanford Veterans Administration Hospital, Palo Alto, CA. The field of view was 12 x 12 cm using a grid of 256/128 pixels per image. The spaces which were dark or not visible in the pre-injection image are now extremely white signaling the presence of the gadolinium chelator portion of NC 100503 present within the nodes. Especially noticable is the transformation on the left side of image from dark spaces to intense white due to the contrast agent. These images are almost too intense and suggest contrast in the supplying lymphatic vessels as well as the nodes themselves.

Figure 15. MRI Lymphography 24 Hours Post Injection of NC 100503

NC 100503; an introperative light imaging contrast agent comprised of both fluorescent dye moieties and gadolinium chelators with molecular weight > 20000 was administered (1 ml) subcutaneously into the dorsal side of the rear paws of rabbits. This image represents the region of the popliteal lymph nodes 24 hours after administration of the contrast agent. The images were taken as spin echo images with TR = 300 ms and TE = 15 ms using a GE Sigma 1.5 Tesla imager at the Stanford Veterans Administration Hospital, Palo Alto, CA. The field of view was 12 x 12 cm using a grid of 256/128 pixels per image. The enhancement of the popliteal lymph nodes has diminished from the 2 hr image but T_1 contrast within the lymph nodes is still visible particularly in the lymph node(s) of the right side of the image.

Figure 16. Fluorescent Imaging of Excised Lymph Node Containing NC 100503

After the 24 hr imaging point, the animal was sacrificed and the popliteal lymph nodes removed under fluorescent light for light imaging assistance during resection. The excised tissue was imaged under a long wavelength fluorescent lamp (i.e., 280 nm< wavelength <400 nm) to provide the image shown here. A fluorescent lymph node can be seen within the tissue exised from the rabbit (see arrow).

Figure 17. Fluorescent Microscopy of a Section of the Lymph Node after Dosing with NC 100503.

Microscopic visualization of the lymph node after conventional tissue fixing processes revealed localization of the fluorescence (long wavelength excitation, 280nm <wavelength< 400 nm) from the contrast agent within the macrophage population lining the sinusoids of the lymph node. While it is impossible to know if the processing solutions "washed out" the contrast agent which was not intracellular, the location of the agent within the sinusoids points to residence within the lymphatic fluid and some uptake by the Mononuclear Phagocytic System (MPS) cells resident within the lymph node.

Example 1

Preparation of $[NH(PEG3400)NHSO_2PcAlCl(SO_3H)_2SO_2]_n$ (NC 100479)

PEG 3400 diamine (Shearwater Polymers, Huntsville, AL ; 0.391 g, 0.115 mMoles) was dissolved in pyridine (75 mL) with magnetic stirring. Approximately 50 mL pyridine were distilled off under nitrogen from an oil bath at 120-130° to dehydrate the PEG, and then the solution was cooled to ambient temperature and $ClAlPc(SO_2Cl)_4$ (prepared from the corresponding acid, Porphyrin Products, Logan, Utah) added (0.111 g, 0.115 mMoles). The solution was stirred for 18 hours at 20° and then refluxed for 30 minutes, after which the solvent was removed on a rotary evaporator at 40° and the residue dissolved in water. This solution was then passed successively through strong acid and strong base (Na form) ion exchange resins to convert the product to the Na salt. Low molecular weight components were removed by diafiltration through a 10,000 membrane (Amicon, Beverly, MA) and the dark blue residual liquid evaporated on a rotary evaporator at 40° to yield a dark blue solid (0.09 g).

Size exclusion HPLC analysis indicated that the product had an average molecular weight of 150,000 , and it had λ_{max} 676 nm (water).

When a solution of this compound in phosphate buffered saline was injected into female immunodeficient mice with HT-29 tumors, 4% of the injected dose was localized in the tumor after one hour.

Example 2

Preparation of $[NHCH_2CH_2NHSO_2PcAlCl(SO_3H)_2SO_2]_n$ (NC 100477)

This was prepared by the same method used in Example 1, but using ethylenediamine (Aldrich, 0.0058 g, 0.10

mMoles) in place of the PEG diamine. The aqueous solution of the product was diafiltered through a 500 membrane, and the dark blue residual solution ion exchanged to the sodium salt, and evaporated to yield a dark blue solid (0.10 g).

Example 3

Preparation of $ClAlPc(SO_2NHPEG5000)_4$

The method used was similar to that described in Example 1, but using PEG 5000 α,ω-bis amine (Shearwater Polymers, Huntsville, AL ; 2.50 g, 0.50 mMoles), pyridine (50 mL, of which about 30 mL were distilled off), and $ClAlPc(SO_2Cl)_4$
(0.10 g , 0.10 mMoles). The solution was refluxed under nitrogen for 30 minutes, and then the solvent was removed. Diafiltration using a 10,000 membrane, collecting the product that did not pass through the membrane, yielded a dark blue solid (0.08 g). It had λ_{max} 676 nm (water). When a solution of this compound in phosphate buffered saline was injected into female immunodeficient mice with HT-29 tumors, 2.5% of the injected dose was localized in the tumor after one hour.

Example 4

Preparation of [Melamine] $[SO_2ClAlPc(SO_3H)_3]_3$ (NC 100478)

$ClAlPc(SO_2Cl)_4$ (0.10 g, 0.10 Moles) was added to dimethylformamide (1 mL) containing diisopropylethylamine (0.1 mL, 0.57 mMoles) and stirred under nitrogen for 1 hour. Melamine (Aldrich, 0.0022 g, 0.017 mMoles) was then added; it was very sparingly soluble in the reaction mixture. The mixture was stirred at ambient temperatures for 4 days, by which time the melamine had dissolved. The solution was poured over ice, and the resultant blue solution was diafiltered through a 3000 membrane. The dark blue solution that did not pass through the membrane was evaporated to yield a dark blue solid, λ_{max} 680 nm (water). When a solution of this compound in phosphate buffered saline was injected into female immunodeficient mice with HT-29 tumors, 0.1 % of the injected dose was localized in the tumor after one hour.

Example 5

Preparation of a stable emulsion of Sudan III

Sudan III (also known as, D&C Red No 17, Solvent Red 23, Cerasin Red) is very water insoluble but soluble in sesame oil, a well known oil for parenteral oil-in-water emulsions (e.g., Intralipid, Lyposin, etc.) and has a λ_{max} of 507 nm. Thus, an emulsion of Sudan III was prepared as follows: A saturated solution of Sudan III in sesame oil was prepared by gently rotating the container over the weekend (approx 72 hr). The oil solution was then filtered through a 5 micron syringe filter followed by a 0.8 micron filter to remove undissolved solid Sudan III. The resulting saturated solution was then emulsified in water at a ratio of 10% "oil" to 90% aqueous surfactant solution using ultrasonic energy followed by microfluidization at approx 14,000 PSI until a constant droplet size was achieved. Droplet size was measured by light scattering using a Horiba 910 light scattering device and a volume weighted average. The resulting emulsions were also sterilized by traditional steam sterilization and the droplet size measured again. The results were:

Formulation	Average Droplet Size(nm) Before Autoclaving	After Autoclaving
1. 1.2% lecithin, 0.3% F68	787	909
2. 1.2% Lecithin, 2% P79	141	199
3. 0.8% Lecithin, 3% P79	122	128

P79, described in Example 2k of PCT/GB95/02109, is a PEG-double ester of molecular weight about 10000 and formula $CH_3(CH_2)_{14}COO(CH_2)_{15}COO((CH_2)_2O)_nCH_3$.

P79 is a polymeric surfactant which appears to add greatly to the ability to make a small emulsion droplet of sesame oil saturated with the Sudan III. The resulting rose colored emulsion is stable on the shelf.

This emulsion (Sudan III) may be injected peri-tumorally to migrate to the regional draining lymph nodes for ease of resection. This is currently done in melanoma and breast cancer. These nodes are important for staging the progress of the disease and planning patient management and is known as sentinel lymph node mapping. This emulsion may also make the histopathology easier and more accurate by staining the healthy tissue thereby making the disease tissue more obvious as a filling defect to the emulsion. Further, the emulsion may be administered iv to effect a marking of the healthy tissue of the liver and spleen and other organs which are MPS rich. This will provide visible contrast between the healthy tissue and diseased tissue, lesions, malformations, etc., for ease in surgical resection. Even areas with low or blocked blood flow would be contrasted with normal vascular beds via the content of the blood of the Sudan III emulsion (P79 has been shown to afford prolonged circulation to liposomes and emulsions in corresponding X-ray contrast formulations).

Example 6
Preparation of nanoparticles of fluorescein

A nanoparticle suspension of Fluorescein was prepared by placing 7.5 ml of milling beads (0.7 mm zirconium silicate) and 0.9 gm of fluorescein into a 15 ml bottle. Using a stock solution of surfactant, the suspension was made up to 3.3 ml in aqueous phase. This was done for each of 3 surfactants: Brij 58, Tyloxapol, and Pluronic F-108. The particle sizing results were:

formulation	Day 3	Day 5	Day 6
F108	247 μm	4.4 μm	194 nm
Tyloxapol	91 nm	---	----
Brij 58	101 nm	---	----

A sterile filtered suspension of fluorescein prepared in this manner was administered subcutaneously to an anesthetized dog with a cannulated thoracic duct to monitor lymph flow and contents. The fluorescein may be detected in the lymph fluid indicating that the dye nanoparticles are passing through the lymphatics thereby marking the lymph nodes as required to aid in the visual identification of lymph nodes for resection and use in cancer staging.

These particles will also function after iv administration in marking tissues rich in MPS cells such that healthy tissue will be marked while disease tissue will remain dark and easily identifiable during surgical resection.

Example 7
Formulation of Indocyanine Green in a Liposome

Indocyanine Green (ICG) was added to a liposome suspension formed from 8.2% lecithin (phosphatidyl choline), 0.8% dimyristylphosphatidylglycerol, and 0.1 % of a nonionic, polymeric surfactant, P-79 which is designed to impart prolonged blood pool residence to the liposome. The phospholipids and the surfactant were mixed in water using ultrasonic energy from a probe sonicator (Bransonic Sonifier 450, 90% duty cycle, output 10). Liposomes were prepared using a Microfluidics M110S microfluidizer at 14,000 PSI and 4 passes through the interaction chamber of the phospholipid mixture. The resulting liposomes were approximately 100 nm in average diameter as determined by light scattering and remained the same size after autoclave sterilization. In addition, these liposomes were able to pass through a sterile filter (i.e., 0.2 micron pore size). Addition of ICG in sufficient amount to make the suspension approximately 7 mg/ml in ICG did not alter the physical characteristics of the liposomal suspensions. After sterilization under a nitrogen atmosphere, these ICG liposomes were stable for at least 6 weeks at room temperature.

Assessment of the spectral properties of the liposomal ICG relative to ICG dissolved in water or saline demonstrated the impact of the liposomal environment. Both the excitation maximum wavelength and emission maximum wavelength were shifted to lower energies (i.e., higher wavelengths) relative to the homogeneous water solutions. In addition, careful measurements of quantum yield demonstrate at least a 4 fold increase in quantum yield of the liposomal ICG relative to the aqueous ICG solutions. Thus, the dose required for light imaging contrast utility of the liposomal formulation of ICG should be significantly less than that required from a homogeneous aqueous solution of ICG.

Example 8

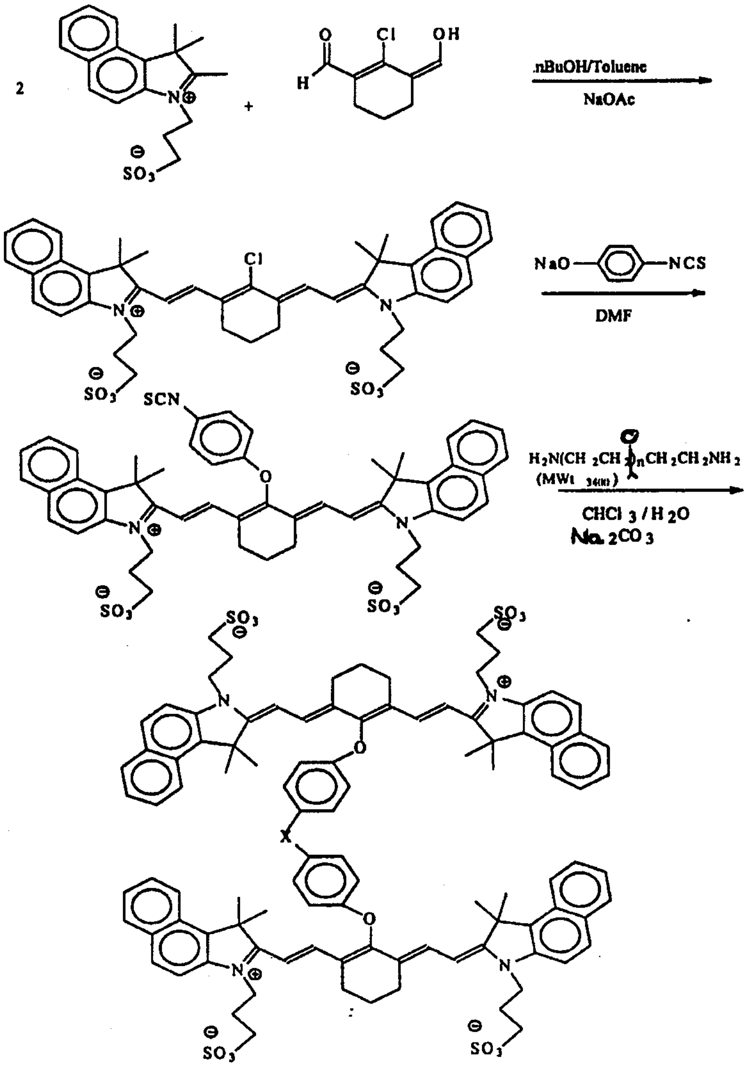
Use of Contrast Media for Enhancement of Laser Doppler Measurement of Blood Flow in the Skin

Approximately 0.5 to 1 hour before the measurements are to be made a sterile solution of a contrast medium containing 5-20 mg of a dye with an absorbing maximum between 300 and 1300 nm (e.g., a Pc dye as described in the foregoing Examples) is administrated by intravenous injection. The actual measurement of blood flow is made with a standard laser Doppler instrument, for example that from Lisca Development AB, Kinkoping, Sweden, that optionally may be modified to incorporate a laser source operating at 830 or 780 nm (Abbot, N.C.; Ferrell, W. R.; Lockhart, J.C.; Lowe, J.G., "Laser Doppler Perfusion Imaging of Skin Blood Flow Using Red and Near-Infrared Sources", *J. Invest. Dermatol.*, **1996**, *107*, 882-886) after the concentration of contrast agent in the blood has stabilized. In this way, highly vascularized, particularly tumorous, structures may be identified.

Example 9

Preparation of 2-[2-[2-(4-isothiocyano)phenoxy-3-[[1,3-dihydro-1,1,-dimethyl-3-(3-sulfopropyl)-2*H*-benz[*e*]indol-2-ylidene]ethylidene]-1-cyclohexen-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulfopropyl)-1*H*benz[*e*]indolium, inner salt, sodium salt, reaction product with PEG 3,400 α,ω-diamine.

The following reaction scheme was used to produce the title compound:



wherein X is NH-CS-NH$(CH_2CH_2O)_nCH_2CH_2$NH-CS-NH).

Example 10

Preparation of 2-[2-[2-(4-isothiocyano)phenoxy-3-[[1,3-dihydro-1,1,-dimethyl-3-(3-sulfopropyl)-2*H*-benz[e]indol-2-ylidene]ethylidene]-1-cyclohexen-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulfoprpoyl)-1*H*benz[e]indolinium, inner salt, sodium salt, reaction product with PEG 10,000 α,ω-diamine.

The title product was produced analogously to that of Example 9.

Example 11

Preparation of Ethyl 3-(N-Acetyl-N-ethylamino)-5-[(5-dimethylamino-1-naphthylsulfonyl)amino]-2,4,6-triiodobenzoate

To a stirred solution of of ethyl 3-(N-acetyl-N-ethylamino)-5-amino-2,4,6-triiodobenzoate (11.6 g, 18.5 mmol) in dry pyridine (75 ml) cooled in an ice bath was added 60% NaH oil dispersion (1.8 g, 46.3 mmol). After the reaction with the sodium hydride subsided, dansyl chloride (5 g, 18.8 mmol) was added. The reaction mixture was stirred at ice bath temperature for 4 hours and then at room temperature for 20 hours. After quenching the reaction with acetic acid (10 ml), the brown solution was concentrated on a rotary evaporator. The resulting brown residue was washed with hexanes and then slurried in water (200 ml). The yellow gummy solid was collected, washed with water, dried and then crystallized from ethanol to give 5.3 g (33%) of bright yellow crystals, mp 238-240°C; ms (FAB) 862 [(M+H),90%]. The ^{1}H-NMR and ^{13}C-NMR spectra were consistent with the desired product.

Analysis: Calculated for $C_{25}H_{26}I_3N_3O_5S$: C 34.86; H 3.05; I 44.20; N 4.88. Found: C 34.91; H 3.02; I 44.53; N 4.74.

Example 12

Preparation of 1-[[2-Diethylamino)ethyl]amino]-4-[(methylamino)methyl]thioxanthen-9-one

This compound was prepared as described in U.S. 5,346,917 (to Miller et al.); Example 5, mp 241-243°C.

Example 13

(see attached synthetic scheme; A → F)

Preparation of α,ω-Bis-(rhodamine B sulfonamide) Analog of Poly(oxyethylene-co-oxypropylene-co-oxyethylene) Block Copolymer with a Block Ratio of 40:20:40 and a Weight Average M.W. of Approximately 14,600

Synthesis of the α,ω-bis-(amino) analog of poly(oxyethylene-co-oxypropylene-co-oxyethylene) block copolymer with a block ratio of 40:20:40 and a weight average M.W. of approximately 14,600 (Compound E):

A total of 50.0 g. of poly(oxyethylene-co-oxypropylene-co-oxyethylene) block copolymer with a block ratio of 40:20:40 and an average molecular weight of approximately 14,600 (Pluronic Surfactant F-108, BASF Corp.) (starting material A, above) was treated with 275 ml of toluene and refluxed for two hours under a Dean Stark trap. The system was then cooled and the trap and its contents (about 25 ml) removed. At this point the reaction mixture was treated with 1.25 ml of thionyl chloride and 0.053 ml of anhydrous dimethylformamide and stirred at 105° for 2 hours. The system was then allowed to stir at room temp. overnight. Next day the reaction mixture was stripped on a rotary evaporator to give 49.35 g of an off-white solid which was readily powdered (intermediate B). In addition to the dominant polyalkylene oxide peaks between 70 and 80 ppm (also seen in the starting Surfactant F-108) the ^{13}C NMR spectrum of the product contains a singlet at 42.69 ppm, consistent with terminal carbons bearing chlorines, and no remaining peak near 61 ppm where the terminal hydroxyl-bearing carbons of Surfactant F-108 show up.

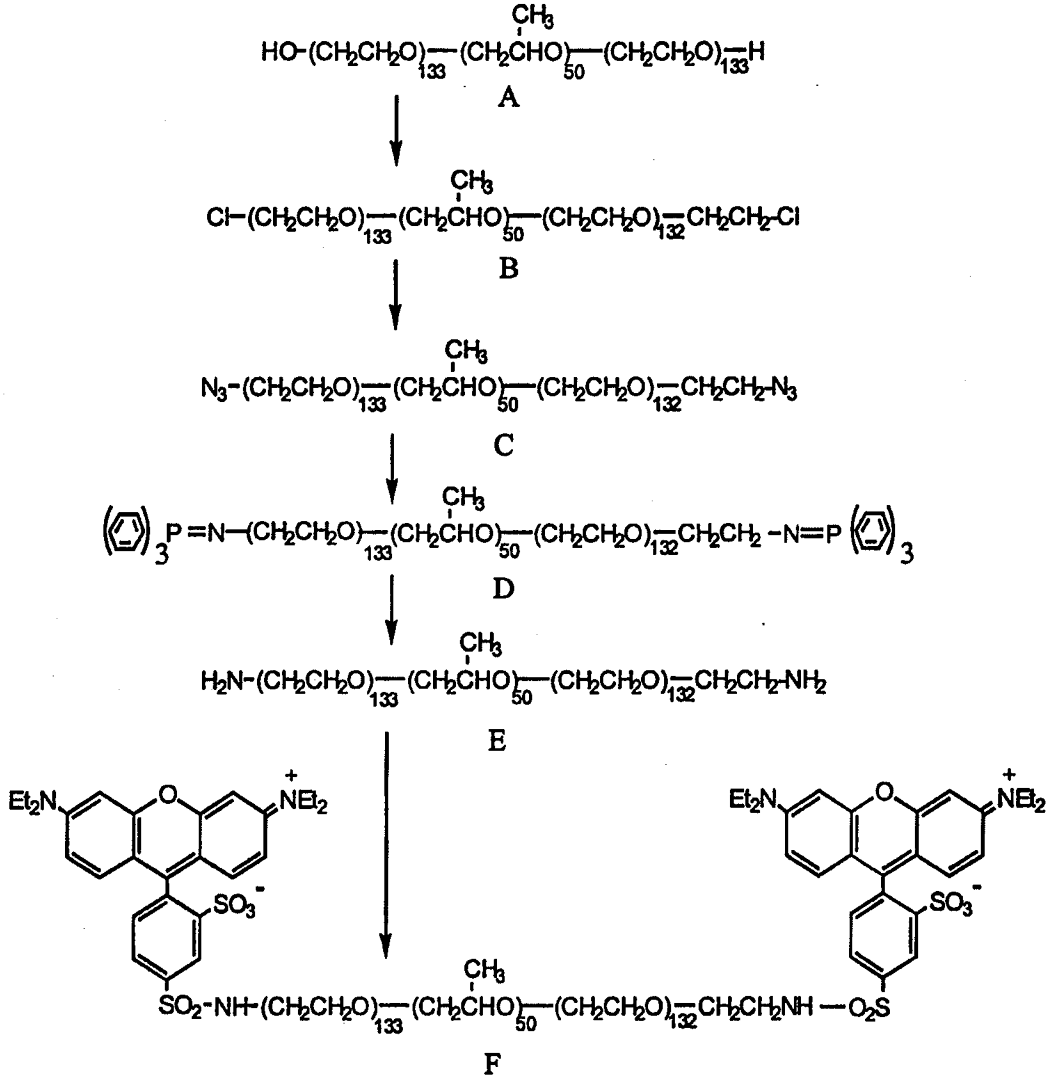
A total of 49.08 g of intermediate B, 0.89 g of sodium azide, and 2.83 g of potassium iodide were treated with 350 ml of anhydrous dimethylformamide and stirred at 100° for 5 hours under dry argon. The reaction mixture was then stirred at room temperature overnight under argon. It was then stripped on a rotary evaporator at 50° to a melt which solidified to a tan solid. The solid was dissolved in 500 ml of distilled water and shaken with 500 ml of chloroform. Upon layer separation (very slow), the aqueous layer was extracted with two 500 ml portions of chloroform. The three chloroform layers were combined and dried over magnesium sulfate. Upon stripping volatiles, 45.58 g of a white solid was obtained (intermediate C). The ^{13}C NMR spectrum of the product contains a singlet at 50.6 ppm, consistent with terminal carbons bearing azides, and no remaining peak near 42 ppm from the starting bis-chloride.

A total of 44.05 g of intermediate C was treated with 3.15 g of triphenyl phosphine and 200 ml of anhydrous pyridine. The reaction mixture was stirred under argon at room temperature. The bis-triphenyl phosphine analog prepared in this reaction (intermediate D) was used directly, without isolation, in the next step of the synthesis.

The reaction mixture from the previous step was treated with 200 ml of 30% ammonium hydroxide (aqueous) and stirred at room temperature for 7 hours. The foaming was vigorous, requiring a very large vessel to avoid foam-over. It was then stripped on a rotary evaporator overnight and the residual solid redissolved in 500 ml of chloroform. Following drying over magnesium sulfate the volatiles were stripped to an off white solid comprising 39.31g. When a phosphorus NMR spectrum of the product indicated that a significant phosphorus signal still remained, a 2.0g sample of the product was treated with 38 ml of 30% ammonium hydroxide (aqueous) and stirred at 60° for 4 hrs. The reaction mixture was then cooled to room temperature, washed with four-40 ml portions of ether, and restripped on a rotary evaporator. The product is an off white waxy solid comprising 1.46 g (intermediate E) This time no phosphorus signal was found in the phosphorus NMR. Also the ^{13}C NMR spectrum contained a peak at 41.78 ppm, consistent with the terminal carbons bearing amines, and had no remaining peak near 50 ppm corresponding to the starting bis-azide.

Synthesis of the α,ω-bis-(rhodamine B sulfonamide) analog of poly(oxyethylene-co-oxypropylene-co-oxyethylene) block copolymer with a block ratio of 40:20:40 and a weight average molecular weight of approximately 14,600.

A total of 1.25 g of the α,ω-bis-(amino) analog of Pluronic Surfactant F-108 from above (intermediate E) was treated with 0.026 g of dimethylaminopyridine and 10 ml of anhydrous pyridine. The resulting solution was treated with 0.12g of rhodamine B sulfonyl chloride (Molecular Probes) and stirred at room temperature under nitrogen overnight. The resulting intensely purple solution was stripped on a rotary evaporator to an intensely purple solid comprising 1.42 g. A total of 1.0 g of the crude product was dissolved in 40 ml of distilled water, filtered through a 0.45 micron nylon filter, and the filtrate diafiltered against distilled water using an 50 ml stirred diafiltration cell (Amicon) containing an Amicon YM-3 (nominal 3000 MW cut-off diafiltration membrane). The diafiltration was continued for 35 turnovers (1,750 ml of diafiltrate removed). Initially, the diafiltrate was intensely purple, but as the purification continued the color intensity diminished till it was virtually colorless at 35 turnovers. The intensely purple retentate was stripped on a rotary evaporator to an intensely purple solid which comprised 0.92 g (final product F). The ^{13}C NMR spectrum of the product contains the dominant polyalkylene oxide peaks between 70 and 80 ppm seen in F-108 and all the subsequent intermediates, as well as a new singlet at 45.69 ppm. No remaining peak near 41 ppm, corresponding to the previous bis-amine intermediate, was observed. Size exclusion HPLC studies indicate a single broad peak with a peak molecular weight of approximately 15,000 based on PEG standards. The compound shows a broad spectral absorbance peaking at 584 nm.


HO-(CH2CH2O)133-(CH2CH(CH3)O)50-(CH2CH2O)133-H
A
Cl-(CH2CH2O)133-(CH2CH(CH3)O)50-(CH2CH2O)132-CH2CH2-Cl
B
N3-(CH2CH2O)133-(CH2CH(CH3)O)50-(CH2CH2O)132-CH2CH2-N3
C
(C6H5)3P=N-(CH2CH2O)133-(CH2CH(CH3)O)50-(CH2CH2O)132-CH2CH2-N=P(C6H5)3
D
H2N-(CH2CH2O)133-(CH2CH(CH3)O)50-(CH2CH2O)132-CH2CH2-NH2
E
Et2N
NEt2
+
O
SO3-
SO2-NH-(CH2CH2O)133-(CH2CH(CH3)O)50-(CH2CH2O)132-CH2CH2NH-O2S
F

Example 14

Synthesis of the Fluorescein Thiocarbamate Derivative of Surfactant T-908

A total of 5.0 g of Tetronic-908 Surfactant (T-908, BASF, Corp., average molecular weight 25,000) (starting material A below) was treated with 0.10 g of dimethylaminopyridine and 50 ml of anhydrous pyridine. The resulting mixture was treated with 0.15 g of fluorescein isothiocyanate (Molecular Probes, Inc.) and stirred at room temperature under nitrogen overnight. The resulting solution was stripped on a rotary evaporator to a yellow-green solid comprising 5.4 g. A total of 5.1 g of the product was dissolved in 200 ml of distilled water, filtered through a 0.45 micron nylon filter, and the filtrate diafiltered against distilled water in a stirred diafiltration cell (Amicon) containing an Amicon YM-3 (nominal 3000 MW cut-off) diafiltration membrane. The diafiltration was continued for 25 turnovers until the diafiltrate was essentially colorless. The retentate was freeze dried to a yellow-green solid which comprised 3.4 g. The ^{13}C-NMR spectrum of the product contains the dominant polyalkylene oxide peaks between 70 and 80 ppm as well as aromatic peaks consistent with the fluorescein moiety. Size exclusion HPLC studies indicate a single broad peak with a peak molecular weight of approximately 25,000 based on PEG standards.

Example 15

Preparation of a Linear Copolymer of Diethylenetriamine-pentaacetic Acid and 1,6- Diaminohexane Terminated with Fluorescein

Amine-terminated Polymeric Ligand

A solution of 4.42g of 1,6-diaminohexane in 68.8 ml of anhydrous dimethylsulfoxide was treated with 13.24 ml of triethylamine and 11.32 g of diethylenetriaminepenta-acetic dihydride. The resulting slurry was stirred under argon overnight. Next morning the moderately viscous polymer dope which had developed was treated with 600 ml of 0.5 M sodium carbonate in water and ultrafiltered to approximately 300 ml in a stirred cell diafiltration unit fitted with an Amicon YM-10 diafiltration membrane (nominal 10 K cutoff). At this point the mode was changed to diafiltration with a 0.5 M aqueous sodium carbonate feed and continued until approximately 1,800 ml of diafiltrate had been produced (approximately 6 turnovers). The feed solution was then changed to distilled water and diafiltration continued until another 1,800 ml of diafiltrate had been produced (6 turnovers). The retentate was then removed and freeze dried to give 7.35 g of a white fluffy polymeric solid. This polymeric ligand was found to have a number average molecular weight of 10000 a weight average molecular weight of 25000 and a dispersity of 2.50.

Fluorescein-terminated Polymeric Ligand

A solution of 3.50g of the above amine-terminated polymeric ligand was dissolved in 29.6 ml of 0.5M aqueous sodium carbonate was treated with 0.72g of fluorescein isothiocyanate, isomer 1, and stirred at room temperature under argon for 2 hours. The bright orange solution which resulted was diluted with 40 ml of distilled water and placed in a stirred cell diafiltration unit fitted with an Amicon YM-10 diafiltration membrane (nominal 10K cutoff). It was then diafiltered until approximately 1,450 ml of diafiltrate had been produced (approximately 18 turnovers). The retentate was then removed and freeze dried. An intensely golden yellow, fluffy polymeric solid comprising 3.00g was obtained. It was found to have a lambda max of 492 nm.

Example 16

Preparation of a the Gadolinium Complex of a Linear Copolymer of Diethylenetriaminepentaacetic Acid and 1,6-Diaminohexane Terminated with Fluorescein

A 2.00 g portion of the linear copolymer of diethylenetriaminepentaacetic acid and 1,6-diaminohexane terminated with fluoresceins described in Example 15 above was treated with 50 ml of distilled water and stirred at room temperature. In a separate vessel 3.13g of gadolinium chloride hexahydrate was dissolved in 31.3 ml of distilled water. The gadolinium chloride solution was then poured slowly into the stirring polymer solution which was then stirred at room temperature for an additional 2 hours. The resulting intensely yellow solution was then rinsed into a stirred cell diafiltration unit fitted with an Amicon YM-10 diafiltration membrane (nominal 10K cutoff). It was ultrafiltered down to a retentate volume of approximately 60 ml following which the mode was changed to diafiltration with a distilled water feed. After 1,400 ml of diafiltrate were produced (approximately 18 turnovers) the intensely yellow retentate was removed and freeze dried. An intensely yellow, fluffy polymeric solid was obtained which comprised 1.09 g. Spectral analysis showed the polymer has a lambda max. (absorption) of 499 nm.

Example 17

Preparation of a Linear Copolymer of Diethylenetriaminepentaacetic Acid and 1,6-Diaminohexane Terminated with Sulfonated Phthalocyanine

A 0.43 g portion of an amine-terminated polymeric ligand made in the same manner as that described in Example 15 above was dissolved in 4.25 ml of 0.5 M aqueous sodium carbonate and stirred in an ice-water bath for 15 min. At this point a total of 0.242 g of powdered aluminumchlorophthalocyanine tetrasulfonyl chloride was slowly added to the cold stirring polymer solution forming a green slurry. The reaction mixture was stirred overnight, allowing the ice to melt and the reaction mixture to come to ambient room temperature. Next morning the reaction mixture had become an intensely green solution. It was rinsed into a stirred cell diafiltration unit fitted with an Amicon YM- 10 diafiltration membrane (nominal 10 K cutoff) with 20 ml of distilled water and diafiltered for approximately 13 turnovers. The retentate was then freeze dried, yielding an intensely green fluffy polymeric solid comprising 0.48 g. Spectral analysis showed the polymer has a lambda max. (absorption) of 650 nm.

Example 18

Preparation of a Linear Copolymer of Diethylenetriaminepentaacetic Acid and 1,6-Diaminohexane Terminated with sulfocyanine

A 0.568 g sample of amine-terminated polymeric ligand prepared in the same manner as that described in Example 15 above is treated with 4.8 ml of 0.5 M sodium carbonate in water and stirred to form a clear solution. That solution is treated with 0.238 g of the monofunctional sulfocyanine dye sold as Cy-5 monofunctional dye by Nycomed Amersham. The reaction mixture is stirred at room temperature for 2 hours, whereupon the clear, red solution is diluted with 20 ml of distilled water and placed in a stirred cell diafiltration unit fitted with an Amicon YM-10 diafiltration membrane (nominal 10K cutoff). It is then diafiltered for 18 hours with a distilled water feed. Approximately 650 ml of diafiltrate is removed (approximately 26 turnovers of diafiltration) following which the retentate is freeze dried.

Example 19

Preparation of the Fluorescein Thiourea Derivative of Surfactant T908 (BASF)

Terminal Amino Derivative of Surfactant T908

A total of 100.0 g of Surfactant T908 (BASF) was treated with 525 ml of toluene and refluxed for an hour under a Dean-Stark trap. The Dean-Stark trap was then removed along with the approximately 25 ml of toluene/water it contained. Upon cooling to room temperature, the reaction mixture was treated with 2.92 ml of thionyl chloride and 0.12 ml of DMF. It was reheated to 105°C and stirred under argon for 3 hours, cooled to room temp, and stripped on a rotary evaporator to a light tan powder comprising 101.99 g.

A total of 100.0 g of this "chloro-T908" intermediate was treated with 2.08 g of sodium azide, 5.98 g of potassium iodide, and 700 ml of DMF. The reaction mixture was heated to 100°C for 6 hours, cooled to room temperature, and then stripped on a rotary evaporator at 69°C. The crude product was dissolved in 900 ml of distilled water and shaken with 1 liter of chloroform. The chloroform layer was collected and combined with two additional 500 ml chloroform washes of the aqueous layer. After drying the combined chloroform layers over magnesium sulfate the chloroform was stripped on a rotary evaporator to give 65.69 g of a light tan frangible solid.

A total of 62.5 g of this "azido-T908" intermediate was dissolved in 300 ml of anhydrous pyridine and treated with 6.56 g of triphenylphosphine. After stirring it at room temperature for 18 hours, the resulting clear solution was treated with 300 ml of 30% ammonia (aqueous) and stirred under argon at 50°C for 5 hours. It was then stripped on a rotary evaporator and subsequently treated with 562.5 ml of DMSO and 2,250 ml of distilled water. The resulting solution was diafiltered for 12 turnovers using a Millipore spiral wound permeator with a nominal 10K cutoff. The final retentate was freeze dried yielding 43.22 g of an off-white powder. A high field ^{13}C NMR of the product showed a peak at 41.78 ppm, consistent with that expected for the methylene adjacent to the terminal amine of the desired product, and showing no aromatic peak in either the ^{13}C or ^{1}H or spectra as well as no peaks in the ^{31}P spectrum indicating the triphenylphosphine adduct has been completely converted to the desired amino adduct.

Terminal Fluorescein Derivative of Surfactant T908

A total of 1.00 g of the above terminal amino derivative of Surfactant T908 was dissolved in 10 ml of distilled water and then treated with 3.2 ml of 0.5M aqueous sodium carbonate. The resulting clear solution was treated with 0.156 g of fluorescein isothiocyanate, isomer I (Aldrich) and stirred at room temperature for 18 hours. The resulting clear intensely orange solution was diluted with 60 ml of distilled water and placed in a stirred diafiltration cell fitted with an Amicon YM-10 (nominal 10K cut-off diafiltration membrane. It was initially ultrafiltered down to a retentate volume of 25 ml and was subsequently diafiltered until 600 ml of diafiltrate had been removed (24 turnovers). The retentate was then freeze dried to a bright orange fluffy polymeric solid comprising 0.43 g. Spectral analysis showed the polymer has a lambda max. (absorption) of 307 nm.

Example 20

Preparation of a Derivative of the Polymeric BASF Surfactant T908 Terminated with Sulfocyanines

A total of 0.30 g of the terminal amino derivative of Surfactant T908 described in Example 19 is dissolved in 3.0 ml of distilled water and treated with 2.9 ml of 0.5M aqueous sodium carbonate. The resulting clear solution is treated with 0.19 g of the sulfoindocyanine dye sold as Cy-7 dye by Amersham and stirred at room temperature for 18 hours. The resulting clear solution is diluted with 6 ml of distilled water and placed in a stirred diafiltration cell equipped with an Amicon YM-10 (nominal 10K cutoff) diafiltration membrane. It is diafiltered until about 250 ml of diafiltrate had been removed (about 20 turnovers). The retentate is freeze dried.

Example 21

Preparation of carboxylic acid substituted benzofluoresceins

2,7-Dihydroxynaphthalene and an X-substituted phthalic anhydride (e.g. X = NO_2, SO_3H, MeO, halogen, CN, COOH, COOR as an ester such as COOMe, ether, thioether, sulphonamide, amide and the like) are mixed and heated together in the melt using excess anhydride as a molten solvent in the presence of a Lewis acid such as zinc chloride to produce the desired compound. This chromophore can be halogenated or sulfonated or carboxylated by the usual methods to produce novel chromophores with added functional groups for solubility modification; for further synthetic elaboration by reduction and displacement reactions; for attachment to anchoring vectors such as antibodies, peptides such as Sta peptide; and for attachment to polymers such as PEG. The material can be radiolabeled with an isotope of iodine by treatment with a source of such isotope such as an isotope as an iodinium ion (eg. iodine chloride or diiodine and the like).

Example 22

Preparation of sulfonic acid substituted benzofluoresceins

2,7-Dihydroxynaphthalene and an X-substituted 2-sulfobenxoic acid cyclic anhydride (e.g. X = NO_2, SO_3H, MeO, halogen, CN, COOH, COOR as an ester such as COOMe, ether, thioether, sulphonamide, amide and the like) are mixed and heated together in the melt using excess anhydride as a molten solvent in the presence of a Lewis acid such as zinc chloride to produce the desired compound. This chromophore can be halogenated or sulfonated or carboxylated by the usual methods to produce novel chromophores with added functional groups for solubility modification; for further synthetic elaboration by reduction and displacement reactions; for attachment to anchoring vectors such as antibodies, peptides; and for attachment to polymers such as PEG. The material can be radiolabeled with an isotope of iodine by treatment with a source of such isotope such as an isotope as an iodinium ion (e.g. iodine chloride or diiodine and the like).

Example 23
Preparation of a PEG derivative of a pH sensitive napthindolecycanine dye (amide linkage via a 4-carboxylic acid of the central cyclohexene ring)

134mg of phenyl-N-phenylphosphoramidochloridate (Aldrich) is added to a solution of 430mg of 4-carboxylic acid-1-hydroxycyclohexene rigidified bis(-3-sulfopropyl-1H-1,1-dimethyl-benzindole) C7 cyanine dye and 0.2ml triethylamine in methylene chloride. The mixture is stirred at room temperature under a nitrogen atmosphere for about 30 mins. To the above solution, dropwise for 30 mins, is added a solution of 2.5g methoxy poly(ethyleneglycol)amine MW 5,000 and 0.07ml triethylamine in methylene chloride. This is stirred at room temperature under a nitrogen atmosphere for approximately one day or until complete reaction was observed. The volatiles were removed from the reaction mixture by placing under reduced pressure. The resulting residue was purified by chromatography (SiO_2, 15-20% methanol in chloroform) giving the desired amide linked cyanine dye.

Example 24
Enhancement of tumor margins with carboxylic acid substituted benzofluoresceins during surgery for breast cancer

An aqueous solution of the dye described in Example 22 is injected into the bloodstream several hours before surgery. The enhanced vascularity of the tumor leads to the selective accumulation of the dye in the tumor. When the tumor is surgically exposed, it appears as a distinctly colored patch.

Example 25

Monitoring of pH in blood with the dye of Example 23

A continuous drip of the dye is administered intravenously. The relative concentrations of the protonated and non-protonated forms of the dye are detected with a monitor attached to a single finger that consisted of a source of infrared light at the absorption maxima of the protonated and non-protonated forms of the dye. A fiber optic cable placed near to the light source conducts light at the same two frequencies to an optical detector. The detector could either measure light that is transmitted through the finger or the light that is reflected back from the finger by scattering sites within the finger. Comparison of the relative intensities of the light at the two frequencies with a standard calibration chart then provides the pH of the blood.

A direct read out of the pH is possible when the detector is attached to a computer that store the calibration information. The output can then be in the form of a pH reading or an audible tone that indicates substantial deviation of the pH from a normal value or from a baseline value for the patient being monitored.

Example 26

Preparation of N-[5-anilino-3-chloro-2,4-(2-ethoxycarbonylpropane-1,3-diyl)-2,4-pentadien-1-ylidene]anilinium chloride

To 34 ml of anhydrous N,N-dimethylformamide, stirred under nitrogen and moderated at 0 to 5 °C by a Dry Ice/isopropanol bath, was added, dropwise over 20 minutes, 28 ml of phosphorous oxychloride. The reaction mixture was allowed to warm for 1 h to 15 °C. To this was then added, dropwise over 5 minutes, a solution of

10 g of ethyl 4-oxocyclohexanecarboxylate (Aldrich Chemical Co.) in 20 ml of methylene chloride. After a brief exotherm had subsided, the reaction mixture was heated to reflux for 2 hours. The solvent was then removed by rotary evaporation, and the dark orange viscous residue was cooled in ice. To this was added, over 35 minutes, a solution of 22 ml of aniline dissolved in 22 ml of ethanol. The addition was accompanied by an evolution of fumes and a rise in temperature that was moderated using an ice-salt bath. After the addition was completed, the viscous reaction product was poured over 250 g of ice containing 25 ml of concentrated hydrochloric acid. This mixture was then allowed to stand in a freezer for 2 days. The crude product was isolated by filtration, washed with water and then with ether, and dried over P_2O_5 under vacuum to give 14 g of solid. This material was used without further manipulation.

Example 27

Preparation of 3-(2,3,3-trimethyl-1H-benz[e]indolio)propanesulfonate

To a magnetically stirred solution of 8.72 g of 1,1,2-trimethyl-1H-benz[e]indole (Fisher Chemical Co.) in 100 ml of anhydrous acetonitrile under nitrogen at room temperature was added 5.09 g of 1,3-propane sultone (Aldrich Chemical Co.) in 3 ml of acetonitrile. The reaction mixture was heated to reflux for 24 hours, and then cooled to ambient temperature. The off-white precipitate was isolated by filtration from the accompanying dark green liquid, washed with 100 ml of acetonitrile and then with 100 ml of ether, and then dried in air to provide 10.24 g of desired compound.

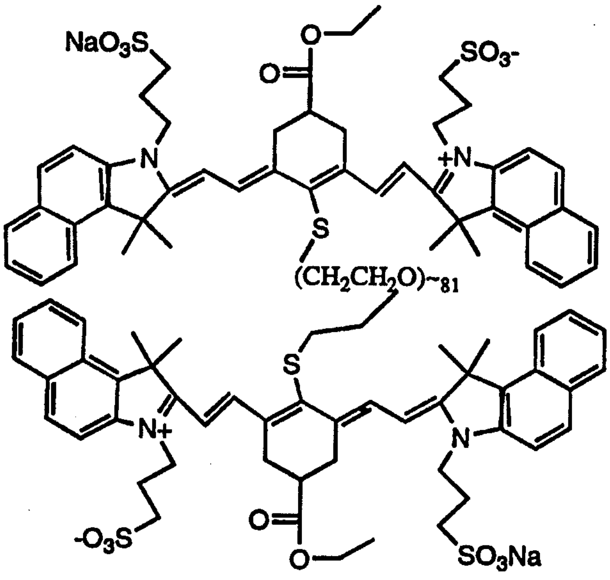
Example 28

Preparation of 2-[2-[2-chloro-3-[[1,3-dihydro-1,1,-dimethyl-3-(3-sulfopropyl)-2*H*-benz[e]indol-2-ylidene]ethylidene]-5-(ethoxycarbonyl)-1-cyclohexen-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulfoprpoyl)-1*H*-benz[e]indolium, inner salt, sodium salt.

A mixture of 1.87 g of N-[5-anilino-3-chloro-2,4-(2-ethoxycarbonylpropane-1,3-diyl)-2,4-pentadien-1-ylidene]anilinium chloride and 4.3 g of 3-(2,3,3-trimethyl-1H-benz[e]indolio)propanesulfonate in 190 ml of n-butanol containing 75 ml of toluene was heated at reflux for one hour with the removal of water. To the mixture was then added 0.65 g of anhydrous sodium acetate, and reflux was continued for another two and one half hours. The solvent was then removed by distillation to a point were crystals began to form. After cooling, the crystals were isolated by filtration, triturated with ethyl ether, and then recrystallized from methanolic ethyl ether to give 1.7 g of the desired compound.

Example 29

Preparation of the bisthioether 2:1 dye:polymer reaction product between 2-[2-[2-chloro-3-[[1,3-dihydro-1,1,-dimethyl-3-(3-sulfopropyl)-2*H*-benz[e]indol-2-ylidene]ethylidene]-5-(ethoxycarbonyl)-1-cyclohexen-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulfopropyl)-1*H*-benz[e]indolium, inner salt, sodium salt and disodium $PEG_{3,400}$-α,ω-dithiolate, Polymer 3

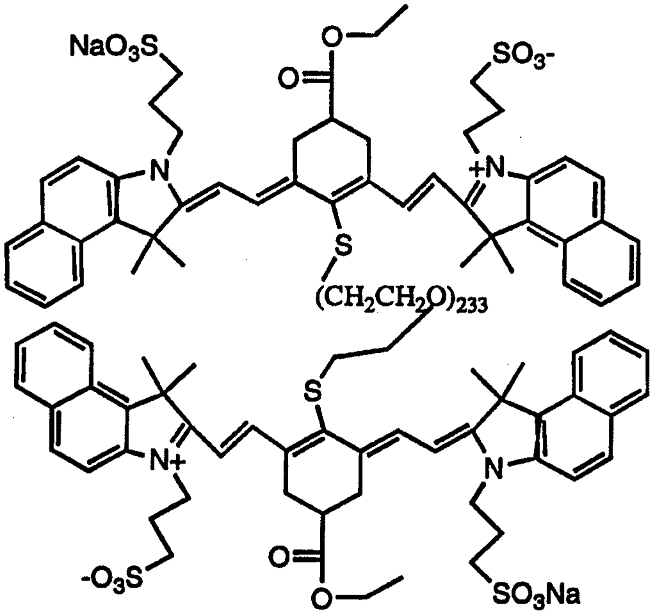


A solution of 1.9 g of 3,400 molecular weight poly(ethylene glycol)-α,ω-dithiol from Shearwater Polymers, Inc. in 8.5 ml of dry and nitrogen-sparged dimethylformamide was treated with 0.1 g of 50% sodium hydride, and then added dropwise under nitrogen at room temperature over 15 minutes to a stirred solution of 0.89 g of 2-[2-[2-chloro-3-[[1,3-dihydro-1,1,-dimethyl-3-(3-sulfopropyl)-2*H*-benz[e]indol-2-ylidene]ethylidene]-5-(ethoxycarbonyl)-1-cyclohexen-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulfoprpoyl)-1*H*-benz[e]indolium in 9 ml of nitrogen-sparged, anhydrous dimethylformamide. After two and one half hours, the reaction mixture was treated with excess carbon dioxide, the solvent was evaporated, and the desired 2:1 dye:polymer adduct was isolated by column chromatography (SiO2: 15% methanol in chloroform).

Biodistribution results are presented in Figures 3A (one hour post-dosing) and 3B (three hours post-dosing).

Example 30

Preparation of the bisthioether 2:1 dye:polymer reaction product between 2-[2-[2-chloro-3-[[1,3-dihydro-1,1,-dimethyl-3-(3-sulfopropyl)-2*H*-benz[*e*]indol-2-ylidene]ethylidene]-5-(ethoxycarbonyl)-1-cyclohexen-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulfoprpoyl)-1*H*-benz[*e*]indolium, inner salt, sodium salt and disodium $\text{PEG}_{10,000}$-α,ω-dithiolate.



A solution of 2.45 g 10,000 molecular weight poly(ethylene glycol)-α,ω-dithiol from Shearwater Polymers, Inc. in 14 ml dry and nitrogen sparged dimethylformamide was treated with 43 mg 50% sodium hydride and 1.5 ml dry dimethylformamide. After about one half hour this solution was added dropwise in one third hour to a nitrogen sparged solution of 0.4 g of 2-

[2-[2-chloro-3-[[1,3-dihydro-1,1,-dimethyl-3-(3-sulfopropyl)-2*H*-benz[*e*]indol-2-ylidene]ethylidene]-5-(ethoxycarbonyl)-1-cyclohexen-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulfoprpoyl)-1*H*-benz[*e*]indolium in 5 ml dry nitrogen sparged dimethylformamide, with stirring. After four hours of stirring under a nitrogen atmosphere, the reaction mixture was treated with excess carbon dioxide followed by evaporation of the solvent. The desired dark green 2:1 dye:polymer adduct was isolated by column chromatography (SiO_2: 20% methanol in chloroform). Absorption maxima in phosphate buffered saline: 814 nm, 744 nm. The mass spectrum had a distribution centered approximately at 12,000 mass units, as expected.

Example 31

Preparation of a Derivative of the Polymeric BASF Surfactant T908 Terminated with Zinc Trisulfophthalocyanine Groups (NC 100526).

This dye was made from the Surfactant T908 amino derivative (2.50g, 0.1mM) by a method analogous to that of Example 20 but using a tenfold excess of zinc phthalocyanine tetrasulfonyl chloride (4.0g, 4.1mM) . The diafiltrate retentate (10,000 MW membrane) was evaporated and freeze dried to yield a dark blue solid, 3.2g, lambda max. 635 nm (shoulder at 671 nm) in water.

Example 32

Preparation of $[PcAlCl(SO_3H)_3SO_2NH]_2$ [PEG 10,000] (NC 100481)

This was prepared by a method analogous to that used in Example 1 but using PEG 10,000 diamine (Shearwater Polymers, 1.0g, 0.1mM) and an excess of chloro-aluminumphthalocyanine tetrasulfonyl chloride (0.217g, 0.22mM). The diafiltrate retentate (3,000 MW membrane) was evaporated to yield a dark blue solid, 0.82g, lambda max. 675 nm in water.

Example 33

Preparation of $ClAlPc[SO_2NH(CH_2)_2N^+(CH_3)_3]_4\ 4Cl^-$

This dye was prepared by a method analogous to that of Example 17, but using chloro-aluminumphthalocyanine tetrasulfonyl chloride (1.25g, 1.29mM) and (2-aminoethyl)- trimethylammonium chloride hydrochloride (Aldrich, 1.0g, 5.7mM, 10% excess). The diafiltrate retentate (500 MW membrane) was evaporated to yield a dark blue solid, 0.44g, lambda max. 677nm in water.

Example 34

Preparation of $ZnPc(SO_3H)_8$

1. Disulfonation of o-toluic acid.

Fuming sulfuric acid (30% oleum, 150mL.) was added to o-toluic acid (50.0g, 0.37 mol.) with stirring, causing an exotherm to 85°C. The solution was heated at 170°C for 6 hours. An additional 50mL of fuming sulfuric acid were added, and the solution heated for an additional 6 hours. The cooled solution was then poured over ice, and the product neutralized to pH 7 with solid calcium carbonate. The precipitated calcium sulfate was filtered, and the solution used for the next step.

2. Oxidation of disulfo-o-toluic acid solution.

The solution from step 1 (100ml.) was stirred and heated almost to boiling, and solid potassium permanganate added in 1g increments until the solution attained a permanent purple color. The excess permanganate was destroyed by the addition of ethanol, the precipitate was filtered, and the resultant pale yellow solution was freed of metal ions by passage through the acid form of a strong acid ion exchange resin (AG 50W - X8, Bio-Rad). The eluate was evaporated to dryness on a rotary evaporator, whereupon it crystallized. The product 3,5-disulfophthalic acid was identified by electrospray MS, (M-H $)^-$ peak at 325 (theor. = 325).

3. Preparation of zinc phthalocyanine octasulfonate. An aqueous solution of 3,5-disulfophthalic acid (6.4g) is neutralized to pH 4 with ammonium hydroxide, and the solution evaporated to dryness. This solid is mixed with urea (10.0g), zinc acetate (1.8g), ammonium chloride (0.8g), ammonium molybdate (0.12g), boric acid (0.12g), and sulfolane (12.5mL), and the mixture heated with stirring under nitrogen at 220°C for 3 hours. The mixture is cooled, the solvent decanted, and an aqueous solution of the residue is chromatographed on cellulose to yield the zinc phthalocyanine octasulfonate as a dark blue powder.

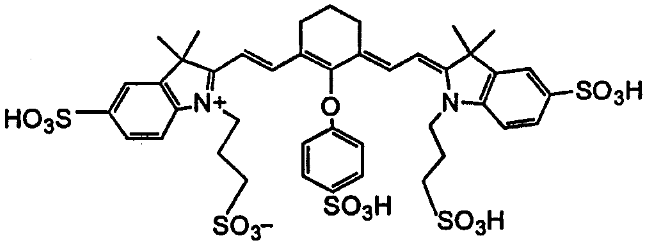
Example 35

Preparation of ^{99m}Tc derivatives of the phthalocyanines of Examples 34

These derivatives are prepared by the method described in Examples 34 and 35 but using sodium pertechnetate ($Na^{99m}TcO_4$, from a generator) together with a reducing amount of hydroxylamine (8 M/M pertechnetate) in place of zinc or aluminum acetate.
Chromatographic separation yields blue 1:1 Pc/Tc complexes, and green 2:1 Pc/Tc complexes.

Example 36

Preparation of cyanine dye containing 5 sulfo groups.



1. Preparation of 1-(γ-sulfonatopropyl)-2,3,3-trimethylindoleninium-5-sulfonate.
The potassium salt of 2,3,3-trimethylindolinium-5-sulfonate was prepared by the method of Mujumdar et al. (Mujumdar, Ernst, Mujumdar, Lewis, and Waggoner, Bioconj. Chem. 1993 4(2) 105). This salt (42.0g, 0.15M), 1,3-propanesultone (25.0g, 0.20M), and acetonitrile (500mL) were refluxed under nitrogen for 4 hours. The pale yellow supernatant liquid was decanted from the precipitated red-blue solid, and the solid washed twice with acetonitrile. It was then stirred with isopropanol (800mL) for 2 days, and the resultant finely divided solid filtered,

washed with isopropanol, and dried under vacuum to give the product (43.5g, 72%).

2. Preparation of cyanine dye.

The above potassium salt (8.0g, 0.02M) and 2-chloro-1-formyl-3- (hydroxymethylene) cyclohex-1-ene (1.7g, 0.01M) were dissolved in a mixture of acetic anhydride (60mL) and acetic acid (40mL) under nitrogen. Diisopropylethylamine (6mL) was then added, and the solution stirred for 2 days. The mixture was then filtered, the solvents evaporated, and the residue treated with 95% ethanol (500mL), stirred, and filtered. The precipitated solid was washed with 95% ethanol (500mL), and dried under vacuum at 40°C. The product was a dark green solid, 5.3g (55%), λ_{max} 780 nm.

FAB-MS was carried out on the acid form of the dye, obtained by ion exchange chromatography, found $MH^+ = 859$ (theor. = 859).

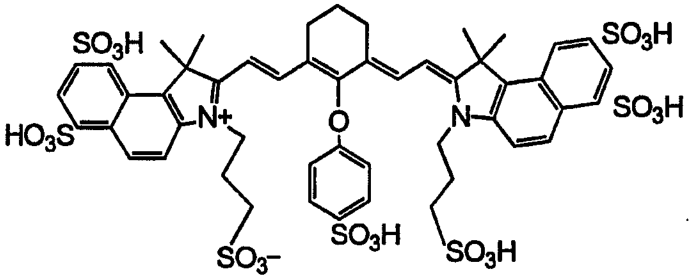
TLC (SiO_2, 40% methanol in methylene chloride) showed a high degree of purity, and no further purification was carried out.

3. Preparation of cyanine dye sulfophenyl derivative.

Phenol-4-sulfonic acid dihydrate (Acros, 0.23g, 1.0mM) in DMF (20mL) was stirred under nitrogen in an ice bath and sodium hydride (Aldrich, 60% dispersion in mineral oil, 0.12g, 3.0mM) in DMF (10mL) added. After stirring for 10 minutes, this solution was added to the product from step 2 (0.83g, 0.9mM) in DMF (35mL) and the mixture allowed to stir for 3 days. The solution was acidified with acetic acid, ether (500mL) added, and the resultant precipitate filtered and washed with ether. The product was purified by chromatography on silica using 40% methanol in methylene chloride as eluant. The dark green eluate was collected and evaporated to yield a dark blue-green solid (0.39g), λ_{max} 773nm. This was purified by dissolution in the minimal amount of methanol and precipitation with excess isopropanol. FAB-MS was carried out on the acid form of the dye (obtained by ion exchange), found MH^+ = 997 (theor. = 997).

Example 37

Preparation of cyanine dye containing 7 sulfo groups.



The potassium salt of 2,3,3-trimethylbenzindoleninium-5,7-disulfonate is prepared by the method of Mujumdar et al. (Mujumdar, Mujumdar, Grant, and Waggoner, Bioconj. Chem., 1996 7(3) 356). Subsequent steps are carried out by the method of Example 37.

Example 38

Preparation of cofacial SiPc - PEG 3400 alternating polymer

Silicon phthalocyanine dihydroxide (Aldrich, 1.0mM), imidazole (Aldrich, 3.0mM), and DMF (2mL) are stirred under nitrogen for 5 minutes. 3-Isocyanatopropyldimethyl-chlorosilane (Gelest, 2.0mM) are added and the mixture stirred for 48 hours. Methanol (5mL) is added, the solution filtered, and the solvents removed under vacuum. The residue is chromatographed on silica, eluting with toluene containing increasing concentrations of methanol. The blue eluate containing the required product is collected and the solvent removed under vacuum.

This product (1.0mM) in isopropanol (10mL) is mixed with a solution of PEG 3400 diamine (Shearwater Polymers, 1.0mM) in isopropanol (10mL) and heated with stirring under nitrogen at 40°C for 5 hours. The solvent is removed under vacuum, and the required product isolated by chromatography on silica.

Example 39

Preparation of cofacial AlPc - PEG 10,000 compound.

Aluminum phthalocyanine hydroxide (Aldrich, 1.0mM), imidazole (Aldrich, 3.0mM), and DMF (2mL) are stirred under nitrogen for 5 minutes. 3-Isocyanatopropyldimethyl-chlorosilane (Gelest, 2.0mM) are added and the mixture stirred for 48 hours. Methanol (5mL) is added, the solution filtered, and the solvents removed under vacuum. The residue is chromatographed on silica, eluting with toluene containing increasing concentrations of methanol. The blue eluate containing the required product is collected and the solvent removed under vacuum.

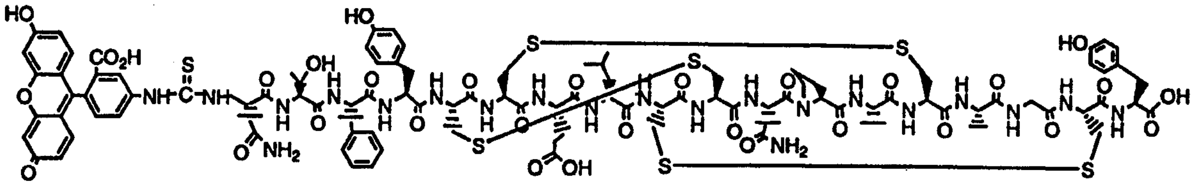
This product (2.0mM) in isopropanol (10mL) is mixed with a solution of PEG10,000 diamine (Shearwater Polymers, 1.0mM) in isopropanol (10mL) and heated with stirring under nitrogen at 40°C for 5 hours. The solvent is removed under vacuum, and the required product isolated by chromatography on silica.

Example 40

Thiourea linked 5-fluorescein to STa (syn. 2): NC 100,506

A solution of 0.12 mg 5-fluorescein isothiocyanate (Molecular Probes) and 0.24 mg heat stable E-coli enterotoxin STa (syn 2, Bachem) (Scott Waldman, US patent 5,518,888) in 0.32 ml 0.1M borate buffer (pH 8.3) was sonicated for one hour with a temperature (external) rise from room temperature to about 40 degrees centigrade. This solution was allowed to sit stoppered at room temperature an additional half hour at which time 0.03 ml of glacial acetic acid was added and mixed into the reaction mixture.

The product had a retention time (RP C18 HPLC) which was longer than the starting peptide and shorter than 5-fluorescein isothiocyanate. Isolation and purification were done using the same HPLC conditions yielding about 0.1 mg yellow product. Ultra violet maxima in 1/1 water/ methanol 10 mM ammonium acetate were: 202 nm (a 0.822), 277 nm (a 0.096), 457 nm (a 0.096), 482 nm (a 0.107). Mass spectrum (electrospray, negative): M-H 2359; M+Na 2381; theoretical M 2360. The conjugate tested positive as a competitive inhibitor of the STa receptor.



NC100506

Example 41

Formulation of Indocyanine Green in a CT-X-ray Diagnostic Liposome

Indocyanine Green (ICG) was added to a CT-X-ray diagnostic liposome suspension (i.e., CTP-10) formed from 8.2% lecithin (phosphatidyl choline), 0.8% dimyristalphosphatidylglycerol, and 0.1 % of a nonionic, polymeric surfactant, P-79 which is designed to impart prolonged blood pool residence to the liposome. The phospholipids and the surfactant were mixed in a solution of 40% iohexol (i.e., Omnipaque) at 80°C using ultrasonic energy from a probe sonicator (Bransonic Sonifier 450, 90% duty cycle, output 10). Liposomes were prepared using an extruder (Avestin, Canada) with 6 x 1 micron pore size filters. The resulting liposomes were approximately 600 nm in average diameter as determined by light scattering and remained the same size after autoclave sterilization. Addition of ICG in sufficient amount to make the suspension approximately 7 mg/ml in ICG did not alter the physical characteristics of the liposomal suspensions. After sterilization under a nitrogen atmosphere, these ICG liposomes were stable for at least 6 weeks at room temperature.

Assessment of the spectral properties of the liposomal ICG relative to ICG dissolved in water or saline demonstrated the impact of the liposomal environment. Both the excitation maximum wavelength and emission maximum wavelength were shifted to lower energies (i.e., higher wavelengths) relative to the homogeneous water solutions. In addition, estimates of quantum yield suggest at least a 4 fold increase in quantum yield of the liposomal ICG relative to the aqueous ICG solutions. Thus, the dose required for light imaging contrast utility of the liposomal formulation of ICG should be signficantly less than that required from a homogeneous aqueous solution of ICG. Further, these liposomes have been demonstrated to provide adequate vascular and liver imaging by CT X-ray examination in rabbits after bolus injection of up to 3 ml/kg. Therefore, either modality could be used to confirm the distribution of the agent in the body after adminstration and to confirm the analysis by one with the other.

Example 42
Multimodal Light + X-ray

Localizations and utility of compositions ethyl 3-(N-acetyl-N-ethylamino)-5-[5-dimethylamino-1-naphthylsulfonyl)amino]-2,4,6-triiodobenzoate and air-exposed surfactant in marking lymph nodes by CT and by visible fluorescence.

A composition comprising a nanoparticle suspension of ethyl 3-(N-acetyl-N-ethylamino)-5-[(5-dimethylamino-1-naphthylsulfonyl)amino]-2,4,6-triiodobenzoate was prepared using Pluronic F108 at 3% (wt/vol %) and 15% (wt/vol % ethyl 3-(N-acetyl-N-ethylamino)-5-[(5-dimethylamino-1-naphthylsulfonyl)-amino]-2,4,6-triiodobenzoate) with 15% PEG 1450 added for tonicity and sterilization. The average particle size of this suspension composition was approximately 200 nm after autoclaving. This suspension composition was sent to the Massachusetts General Hospital, Center for Imaging and Pharmaceutical Research (CIPR) for CT imaging in rabbits. The rabbits were dosed at 2 x 0.5 ml in the dorsal side of the front and rear paws with the following levels of lymph node opacification measured at various times post injection:

Lymph Node Enhancement at Times post injection*

	Times		
Location	2.5 hr	24 hr	7 days
Axillary	2.38	2.07	1.44
Popliteal	2.28	4.29	1.50

* These values have been converted to mg I/ml in the lymph node. 1.5 is considered background in these measurement.

Additionally, one animal was sacrificed at 24 hr post injection such that the popliteal lymph node could be resected for visual inspection. This node, when illuminated with UV light, gave a green fluorescence from the dansyl-containing composition.

Example 43

Multimodal Light + MRI

Preparation of a fluorescein-terminated linear copolymer of a PEG-diamine and DTPA

A copolymer is prepared from one part of PEG 3,400-diamine (Shearwater Polymers) and one part DTPA anhydride (Aldrich) as described in Example 15. Before aqueous workup, the reaction mixture is treated with an additional 0.1 part of PEG 3,400-diamine, and the reaction mixture is heated for an additional 24 hours to end-cap the polymer with amine groups. As in Example 15 during workup of the reaction mixture the diafiltrate is made basic with sodium carbonate, and the diafiltered polymer solution is treated with excess fluorescein isothiocyanate which reacts with free amine groups in the polymer.

Example 44

Preparation of a gadolinium complex of a fluorescein-terminated linear polymer of PEG-diamine and DTPA

A gadolinium complex of the polymer prepared in Example 46 is prepared according to the method of Example 16 by treatment with gadolinium ion. This polymer can be used as a contrast agent in MR imaging of tumors, and can be detected by emission of the attached fluorescein chromophores during intraoperative procedures.

Example 45
Multimodal Light + Nuclear Imaging

Preparation of an ^{111}In complex of a fluorescein-terminated linear copolymer of PEG-diamine and DTPA

A volume of radioactive indium chloride ($^{111}In_{49}Cl_3$; $L_{1/2}$ of 2.83 days; in 0.04 M hydrochloric acid at a specific activity of >5 Ci/mg; Amersham-Mediphysics) is neutralized using two volumes of 0.5 M sodium acetate, pH 6.0. The neutralized ^{111}In is added to a 1.0 mL aliquot containing the fluorescein-terminated linear copolymer of PEG-diamine and DTPA prepared in Example 46 and dissolved in 50 mM sodium acetate buffer containing 150 mM sodium chloride at pH 5.6 The labelling is allowed to proceed for one hour and then the reaction mixture is loaded onto a PD-10 chromatography column which has been prewashed and equilibrated in a buffer containing 50 mM sodium phosphate with 150 mM sodium chloride pH 7.4 (PBS). The sample is eluted from the column with PBS. Fractions of radiolabeled polymer are collected, assayed for radioactivity, and pooled.

This polymer can be used as a contrast agent in gamma imaging of tumors, and can be detected by emission of the attached fluorescein chromophores during intraoperative procedures.

Example 46
Multimodal Light + Ultrasound

Preparation of a fluorescent ultrasound contrast agent

N-1(1,2-dipalmitoyl-sn-glycero-3-succinyl)-succinimide is prepared according to the method of Unger (WO 96/40285; see Example 4 therein) and is treated with fluoresceinamine isomer 1 (Aldrich) to provide the fluorescein labelled lipid by displacement of the hydroxysuccinimide group. This material is converted to an ultrasound contrast agent according to the method Unger in WO 96/40285 (see Example 30 therein), typically by using less than 1% by weight of the fluorescent lipid in the formulation.

The presence of this contrast agent can be detected using ultrasound imaging because of its ultrasonic echogenicity and also by light imaging techniques such as confocal microscopy during intraoperative procedures such as during open heart surgery where the integrity of blood flow through blood vessels can be monitored.

Claims:

1. In a method of treatment of the human or animal body to remove diseased tissue therefrom, wherein an effective amount of a dye compound is administered to said body and allowed to accumulate at diseased tissues or cells therein and wherein diseased tissue or cells at which said dye compound has accumulated is removed or destroyed in situ, the improvement comprising using as said dye compound a physiologically tolerable dye compound being a polysulphonic acid the molecular structure whereof comprises at least one chromophore and at least 5 sulphonic acid groups, or a derivative thereof.

2. A method as claimed in claim 1 wherein said dye compound is a cyanine, phthalocyanine or naphthalocyanine.

3. A method as claimed in claim 2 wherein said dye compound contains at least 6 sulphonic acid groups per molecule.

4. A method as claimed in claim 1 wherein said dye compound is a cofacial polymer of a phthalocyanine or naphthalocyanine.

5. A method as claimed in any of the preceding claims wherein the distance of said tumor or cells to the surface of said body is such that said dye is detectable at the surface of said body.

6. A method as claimed in any of the preceding claims wherein said dye accumulates at a sentinel lymph node.

7. A physiologically tolerable dye compound being a polysulphonic acid the molecular structure whereof

comprises at least one chromophore and at least 5 sulphonic acid groups, or a derivative thereof.

8. A compound as claimed in claim 7 wherein said dye compound is a cyanine, phthalocyanine or naphthalocyanine.

9. A compound as claimed in claim 8 wherein said dye compound contains at least 6 sulphonic acid groups per molecule.

10. A compound as claimed in claim 7 wherein said dye compound is a cofacial polymer of a phthalocyanine or naphthalocyanine.

11. A pharmaceutical composition comprising a physiologically tolerable dye compound being a polysulphonic acid the molecular structure whereof comprises at least one chromophore and at least 5 sulphonic acid groups, or a derivative thereof, together with at least one physiologically tolerable carrier or excipient.

12. A pharmaceutical composition as claimed in claim 11 further comprising an imaging/therapeutic agent.

13. A pharmaceutical composition as claimed in claim 11 further comprising a second physiologically tolerable dye compound being a polysulphonic acid the molecular structure whereof comprises at least one chromophore and at least 5 sulphonic acid groups, or a derivative thereof, and wherein said second physiologically tolerable dye compound may be identical or different to the first physiologically tolerable dye compound.

14. A physiologically tolerable dye compound being a compound that (i) is presented in particulate form, (ii)

is a polychromophore containing at least one sulphonic acid group, (iii) is a cofacial polychromophore, (iv) is a vector-attached chromophore or polychromophore, (v) is a polysulphonic acid compound containing at least 5 sulphonic acid groups or derivatives thereof, (vi) is a polychromophore, the chromophoric groups of which are linked together by a polymer, (vii) is a polymer or surfactant molecule to which chromophoric groups have been attached, or (viii) meets two or more of the foregoing criteria for use in surgical or diagnostic techniques.

15. A dye compound as claimed in claim 14 wherein the surfactant to which chromophoric groups have been attached is one of the Tetronic or Pluronic polymeric surfactants.